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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :		(11) International Publication Number:	WO 90/02339
G01N 33/72, C12Q 1/28	A1	(43) International Publication Date:	8 March 1990 (08.03.90)

(21) International Application Number: PCT/US88/02806

(22) International Filing Date: 16 August 1988 (16.08.88)

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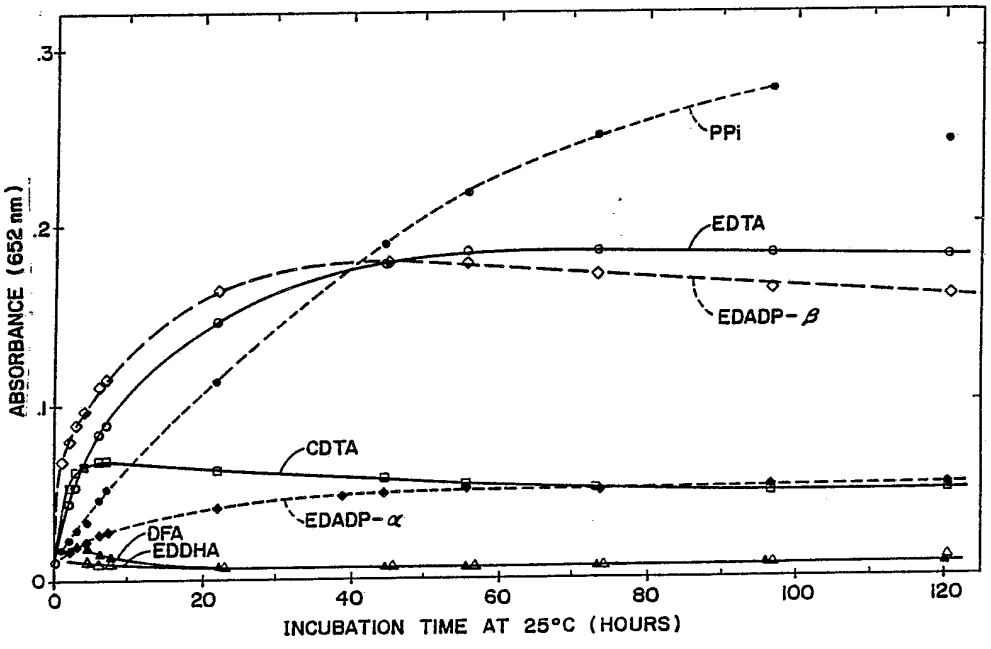
(81) Designated States: AT (European patent), AU, BE (European patent), BG, CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, RO, SE (European patent), US.

Published
With international search report.

(54) Title: STABLE INDICATOR SOLUTIONS FOR DETECTION OF PEROXIDATIC ACTIVITY

(57) Abstract

Disclosed are (a) aqueous indicator solutions useful for detection and quantitation of peroxidatic activity, containing a hydroperoxide and a chromogenic organic electron donor of the benzidine family, which possess the novel property of developing negligible color and maintaining approximately full sensitivity during storage at 0-40°C on a time scale minimally of six months; (b) indicator solutions for peroxidatic activity wherein the chromogen is o-phenylenediamine and storage stability is minimally for one to two days at room temperature or two weeks at 5°C; (c) certain chelators which promote these storage stability goals for o-phenylenediamine and for the benzidine compounds; and (d) processes for preparing such indicator solutions, comprising measures for minimizing contamination by oxidatively active transition metals. The resulting solutions increase the simplicity and reliability of a wide array of chemical, biological, and clinical tests, where either the analyte is a peroxidatic catalyst or the analyte is detected through the specific binding of another molecule, linked directly or indirectly to a peroxidatic catalyst.



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Title

STABLE INDICATOR SOLUTIONS FOR DETECTION OF PEROXIDATIC ACTIVITY

Background of the Invention

Field of the Invention

This invention relates to aqueous mixtures of hydroperoxides and organic chromogenic electron donors, which are used for the detection and quantitation of peroxidatic catalytic activity in a wide array of chemical, biological and clinical tests.

Description of Background and Related Art

Peroxidatic oxidation usually occurs according to one or the other of the following reaction schemes: $AH_2 + ROOH \rightarrow A + ROH + H_2O$; $H_2O + AH_2 + ROOH \rightarrow AH_2^{+2} + ROH + 2OH^-$; in which AH_2 is an electron donor and ROOH is a hydroperoxide. The catalysts for peroxidatic oxidation are of five main classes: certain transition-metal ions and their complex ions, hematin compounds, hemoproteins, the peroxidase enzymes, and the catalase enzymes. Such catalysts are said to have peroxidatic activity. A hydroperoxide also can react with itself to produce molecular oxygen and the corresponding alcohol. This reaction, called dismutation, is catalyzed by the same classes of compounds as catalyze peroxidatic reactions. Many of the electron donors for peroxidatic reactions also can be oxidized by molecular oxygen. These oxidation reactions also tend to be catalyzed by transition metal ions.

The detection of peroxidatic activity has broad application in analytical, biological, and clinical chemistry. Typically, the test consists of exposing a mixture of a hydroperoxide and a chromogenic electron donor to a sample containing the suspected analyte, which either is the catalyst or is associated with the catalyst. If the analyte is present, peroxidatic reaction will

occur; and a color, characteristic of the chromogen used, will be visible in a few seconds to a few hours. Tests in which the analyte is the peroxidatic catalyst include the detection of heme or hemoglobin in forensic specimens or in gastric fluid, feces, urine, or cell-free blood plasma or serum. Tests in which the analyte is associated with the peroxidatic catalyst include enzyme-linked specific binding assays (e.g., immunoassays or nucleic acid hybridization assays) where a peroxidase enzyme is attached to a molecule which directly or indirectly binds to the analyte in a specific manner and in such a way that catalytic activity would be seen only when analyte is present. With either approach, the amount of soluble product of the catalyzed peroxidatic oxidation of the chromogenic electron donor is approximately proportional to the amount of analyte present. The analyte can then be quantitated visually, spectrophotometrically, or colorimetrically by way of comparison with signals obtained from standard analyte solutions.

The detection of catalysts for peroxidatic activity is also applicable to the localization of analytes bound to solid supports, such as filter-trapped enzyme immunoassays, cytochemical smears, histochemical sections, electrophoretic gels, or blots of electrophoretic gels. Here also, the peroxidatic catalyst may be the analyte or be associated with the analyte. The former application is exemplified by locating myeloperoxidase activity in white blood cells in a cytochemical or histochemical preparation. The latter, more common, application includes assays in which the catalyst is a peroxidase enzyme bound to an immunological or nucleic acid probe for a specific analyte which is immobilized on a filter membrane. For example, Western blots can be used for the immunological detection and identification of gel-electrophoresed and membrane-bound antigens or of antibodies which bind to such antigens; and Southern and Northern blots can be used for the nucleic acid hybridization detection and identification of gel-electrophoresed and membrane-bound specific DNA and RNA sequences, respectively. In these cases, the product of the catalyzed peroxidatic oxidation of the chromogenic electron donor remains localized at the site where analyte is bound.

Of the peroxidase enzymes which might be attached to molecules which directly or indirectly bind analyte, horseradish peroxidase (HRP) has proven to be especially useful. It is safer to use than radioactive tags and

more sensitive than chromophores and fluorophores used to label binding moieties. It has a higher catalytic activity or more convenient chemical properties than other enzymes commonly used as analytical labels. It is inexpensive to produce, stable under relatively harsh conditions, and easy to attach chemically to other proteins and to nucleic acid probes. Four classes of chromogenic electron donors are preferred in the detection of peroxidatic activity: (a) the benzidine compounds, including benzidine, 3,3'-dimethylbenzidine (o-tolidine), 3,3'-dimethoxybenzidine (o-dianisidine, or oDAD), 3,3'-diaminobenzidine (DAB), 3,3',5,5'-tetramethylbenzidine (TMB), and 2,7-diaminofluorene (DAF, a benzidine analogue); (b) the phenylene diamines, especially o-phenylene diamine (oPD); (c) 2,2'azino-di(3-ethyl-benzthiazoline sulfonate) (ABTS), and (d) 3-amino-9-ethylcarbazole (AEC). Copending and commonly owned U.S. Patent Application Serial No. 896,677, filed August 20, 1986, discloses methods of extending the versatility of the benzidine chromogens by showing how the solubility of the colored oxidation product can be controlled by the nature and concentration of the anions present.

In a variation on the theme of peroxidatic detection, peroxidase activity is used to indicate the presence of oxidase activity. Oxidase enzymes catalyze the reduction of molecular oxygen to hydrogen peroxide by electron donors, such as glucose, which usually are not chromogens. The hydrogen peroxide is detected or quantitated by reacting it with a chromogenic electron donor in the presence of a peroxidase enzyme, usually HRP, added to the test sample by the analyst. The indicator solution for peroxidase-linked oxidase assay resembles that for peroxidase assay except that no hydroperoxide is added and the buffered pH is chosen for compatibility with oxidase as well as peroxidase activity.

A consistent problem in chromogenic assays of peroxidatic activity has been the spontaneous tendency of hydroperoxides and chromogenic electron donors to react before the addition of a peroxidatic catalyst. This background, or baseline, color formation limits the usable lifetime of indicator solutions often to no more than a few hours. It can raise the detection limit of an assay for peroxidatic catalytic activity, as one cannot confidently verify the existence of a quantity of catalyst which generates color intensity equal to or less than that which appears spontaneously without added catalyst. Less severe

but still significant has been the tendency of dissolved chromogenic electron donors to be oxidized to colored products by dissolved oxygen even in the absence of a hydroperoxide, and the spontaneous tendency of the dissolved hydroperoxide to undergo dismutation.

Practically speaking, there are two goals in the formulation of storage-stable activity indicator solutions. One is to achieve negligible color formation over the useful lifetime of a solution, where "negligible" implies invisibility when the solution is used in a test with a visual readout (e.g., in cytochemistry, histochemistry, immunoblotting, nucleic acid probes blot procedures, and filter-binding immunoassays) and the lowest possible absorbance, preferably below 0.01 absorbance units, in assays with a spectrophotometric readout. Visibility is linked to absorbance in that an absorbance near 0.03 approximates the visual threshold over most of the visible electromagnetic spectrum. The second practical goal is to retain full sensitivity to peroxidatic activity over the useful lifetime of an indicator solution, where the quantitative definition of "full" will depend on the precision of a given assay format. For example, a test with a visual readout might tolerate a 30-50% drop in sensitivity before detection limit would be affected practically, whereas an instrumental assay might suffer unacceptable decline in performance with more than a 20-30% drop in sensitivity. In most cases, an indicator solution which meets the stability criteria for an instrumental assay will surpass the requirements for tests with visual readout. These two performance goals must be met separately because there exist spectrophotometrically "silent" modes of indicator solution degradation, such as hydroperoxide dismutation and the adsorption of chromogen to the container walls; and visibly colored indicator solutions still may be fully sensitive to peroxidatic catalysts.

Also from a practical perspective, two classes of useful lifetime for indicator solutions can be envisioned to be most significant. Storage stability on the scale of a year or more suffices for manufacture and marketing for mass consumption, achieving economies of scale in supply and high inter-day precision over a broad user population. It is especially fortunate if such stability is indifferent to temperature over the 0-40C range, so that minimal precautions in shipping and storage are necessary. However, particularly labile chromogens, for which this first lifetime criterion is unrealistic, may still

benefit from achievement of a useful lifetime of approximately eight hours or one week for the combination of chromogen with hydroperoxide, especially if separate formulations of chromogen and hydroperoxide are stable for much longer periods. Then the individual user will have to prepare fresh indicator solution only once a day or once a week, with the benefits of convenience and improved reliability and precision. If a buffered hydroperoxide solution can be stabilized without chromogen for periods of a year or more, some advantages in mass marketing, convenience, and inter-user precision are still obtained despite the need for users to add chromogen on a more frequent schedule.

As evidenced in the scientific literature, the general experience has been that in chromogenic assays of peroxidatic activity, the hydroperoxide and the chromogenic electron donor must be mixed immediately before use because they spontaneously darken on the time scale of minutes to a few hours. Van Duijn [(1955) Receuil des Travaux Chimiques des Pays-Bas 74:771-778] observed the spontaneous coloration of a benzidine-H_2O_2 histochemical staining reagent within 30 minutes of preparation. In neurohistochemical use of TMB and H_2O_2 to visualize HRP location, Mesulam [(1978) J. Histochem. Cytochem., 26:106-117] detailed the non-specific reaction of the substrates to give a blue deposit on histochemical sections or a green color in the incubation medium. Morrell et al. [(1981) J. Histochem. Cytochem., 29:903-916] confirmed these results and further noted that if labware was not adequately cleaned, TMB solutions would turn blue before addition of H_2O_2. Imberty et al. [(1984) Plant Science Letters, 35:388-393] noted that an assay solution containing TMB and H_2O_2 spontaneously developed color, and specified that it be prepared fresh for each experiment. Liem et al. [(1979) Anal. Biochem., 98:388-393] indicated that aqueous acidic solutions of TMB spontaneously developed color even before the addition of peroxide, apparently because of air oxidation. Broyles et al. [(1979) Anal. Biochem., 94:211-219] reported that benzidine-H_2O_2 solutions should be used within two hours of preparation, turning brown if stored overnight, even at 4C. White [(1977) J. Chrom., 138:220-222] advised separate application of stock solutions of chromogen and H_2O_2 used to visualize iron porphyrins such as heme on solid surfaces such as chromatographic supports. Bos et al. [(1981) J. Immunoas., 2:187-204] reported on the use of TMB for enzyme immunoassays and advised adding H_2O_2 to TMB or oPD solutions immediately before use.

Gallati and Pracht [(1985) J. Clin. Chem. Clin. Biochem., 23:453-460] stated that a fresh TMB-H_2O_2 solution is usable for 2-3 hours, and Porstmann et al. [(1985) J. Clin. Chem. Clin. Biochem., 23:41-43] described a significant background reaction of oPD and H_2O_2 in the absence of added peroxidatic catalyst.

U.S. Patent No. 4,503,143 described the rapid "uncatalyzed" reaction of oPD and H_2O_2 and the much slower reaction of TMB and H_2O_2. These limitations of complete peroxidatic activity indicator solutions supported the rationale for the invention claimed in that patent, namely, the separate formulation of TMB in an organic solvent and H_2O_2 in the assay buffer for long-term storage stability, followed by mixing of the two shortly before use in an enzyme immunoassay (the only analytical context specified in U.S. Patent No. 4,503,143). This approach to the practical formulation of peroxidatic activity indicator solutions remains the accepted state of the art, despite the following efforts to stabilize chromogen-peroxide mixtures. (1) U.S. Patent No. 4,596,770 described the uncatalyzed oxidation of TMB by H_2O_2 and attempted to decrease the severity of this "substrate drift" with a water-miscible organic cosolvent, N-methyl pyrrolidone. This cosolvent reduced the rate of coloration by only 50-75% relative to two alternative cosolvents, and all three indicator solution formulations showed absorbance values high enough to give visible color within two to three hours of preparation. There was no pretense that N-methyl pyrrolidone might in and by itself lead to practical storage stability for peroxidatic activity indicator solutions. (2) Attempts to stabilize aqueous formulations of H_2O_2 plus organic electron donors by the addition of water-soluble polymers were described in U.S. Patent No. 4,615,972. The stabilizing effect of such an inclusion was shown for only approximately one week and only with one of the least sensitive chromogenic electron donors, 4-chloronaphthol, which would be expected to undergo spontaneous oxidation relatively slowly. (3) Weaver and Powell [International Patent Application WO86/05207] disclosed the stabilization of indicator solutions containing TMB and H_2O_2 for as long as approximately two months by inclusion of ß-cyclodextrin, at the cost of a significant reduction in indicator sensitivity to peroxidatic activity. Stability was judged only in terms of retained sensitivity; whether such solutions remained color-free was not shown. (4) Buck and Gibson [European Patent Application No. 86/305828.5] claimed alkaline (pH 7.0-

9.5) liquid (preferably aqueous) formulations of TMB as showing improved long-term storage stability, without disclosure of supporting data. Such a solution still requires the addition of a hydroperoxide before use to indicate peroxidatic activity, and so does not address the technical challenge of how to achieve practical stability of liquid chromogen-hydroperoxide mixtures.

The background reaction of hydroperoxides and chromogenic electron donors in the absence of added peroxidatic catalyst appears to be enhanced by trace amounts of those transition metals known to cycle easily between at least two oxidation states, such as Mn, Cr, Fe, Co, Ni, Cu. These metals (especially Fe) are so ubiquitous in the environment that strenuous efforts are necessary to prevent them from contaminating the equipment and reagents used to prepare and contain peroxidatic activity indicator solutions. Other transition metals, such as Mo, Ru, Rh, W, and Re, also form ions capable of catalyzing peroxidatic and catalatic reactions; but they normally are such rare components of chemical systems that contamination with them is not practically important. It is likely that all of the reported beneficial effects of 1-methyl-2-pyrrolidone, water-soluble polymers, ß-cyclodextrin, and high pH relate in some way to the supply or the inhibitory sequestration of trace catalytic transition-metal-ion contaminants, principally Fe(III).

Transition metal ions have been heavily studied as catalysts of hydroperoxide dismutation and have been implicated in non-catalatic peroxidatic catalysis as well [reviewed by Brown, et al. (1970) Progress in Inorganic Chemistry, 13:159-204; see also Walling (1970) Accounts of Chemical Research, 8:125-131; Sigel, et al. (1979) Inorganic Chemistry, 18:1354-1358]. Chelators influence the rates of peroxidatic and catalatic catalysis by transition metal ions, sometimes accelerating and sometimes inhibiting the reaction in a manner which depends on the reaction being catalyzed, the specific transition metal ion added, and the specific chelator used. For example, EDTA has been used to stabilize H_2O_2 at high pH [Koubek, et al. (1963) J. Amer. Chem. Soc., 89:2263-2268], yet Fe(III)-EDTA complexes are clearly catalatic and peroxidatic catalysts [Walling, supra]. Several chelators have been observed to stimulate or inhibit non-catalyzed lipid peroxidation in a concentration-dependent manner, with only one of them, desferrioxamine B (DFA), being inhibitory at all concentrations tested [Gutteridge, et al. (1979) Biochemistry Journal, 184:469-472]. The ability

of four chelators, DFA, bathophenanthroline sulfonate (BPS), diethylenetriaminepentaacetic acid (DTPA), and ethylenediaminetetraacetic acid (EDTA), to retard iron-catalyzed auto-oxidation of several catecholamines depended on the catecholamine and the chelator. In this case, EDTA and BPS were capable of accelerating the reaction; only DFA and DTPA were consistently inhibitory, usually to similar degrees. [Heikkila and Cabbat (1981) Biochemical Pharmacology, 30:2945-2947]. Efforts to control metal ion contamination in indicator solutions for peroxidatic activity have been described in the prior art. Mesulam, supra, stated that metal ions could cause the auto-oxidation of TMB (i.e., its reaction with O_2) and advised that the water used to formulate reagent solutions and the incubation glassware be free of excess metal ions. However, Morrell et al. supra, stated that while carefully cleaned labware was important for the prevention of chromogen coloration before peroxide addition, this precaution did not eliminate a significant background reaction after peroxide addition. Furthermore, chelating agents are described as constituents of indicator solutions for peroxidatic activity. Liem et al. supra, Bos et al. supra, and Gallati et al. supra, used citrate-containing buffers but betrayed no intent to sequester contaminating metal ions. Van Duijn, supra, clearly understood that the background reaction of a benzidine-H_2O_2 indicator solution was transition-metal-ion catalyzed, extending usable lifetime from 30 minutes to one day by adding hydroxylamine (thought to be a weak chelator) and to one week by adding EDTA. Porstmann et al. supra, cited the addition of EDTA to reaction solutions containing H_2O_2 and oPD, but stated that it did not reduce their nonenzymatic reaction and concluded therefrom that metal-ion catalysis does not occur in this case. European Patent Application 123,902, filed March 27, 1984, described the inclusion in the assay reagent of chelating agents for the sequestration of heavy metals, and used a citrate buffer. PCT Patent Application WO86/04610 disclosed that chelators could drastically reduce the nonenzymatic development of color in chromogen-peroxide mixtures, but the solutions did not remain colorless after one day at room temperature.

In summary, the prior art has been unable to solve the persistent problem of the spontaneous tendency of hydroperoxides and electron donors to react before the addition of peroxidatic catalysts, especially for the practical case in which it is desired to store such solutions for many months without

significant coloration or sensitivity loss. The various separately suggested remedies of metal-free solvent, clean glassware, and the addition of organic cosolvents, water-soluble polymers, or chelating agents have had only partial and incompletely documented success. In those instances where the background reaction was monitored directly, aqueous solutions of hydroperoxides and organic chromogenic electron donors appear to have remained colorless for, at the most, one week. The greatest reported success in preserving the sensitivity of a TMB-H_2O_2 indicator solution (for up to two months) was obtained by adding a component which reduced analytical sensitivity [Weaver and Powell, supra]. A mass-marketable indicator solution will require at least a several-fold extension of this storage lifetime, with clear evidence of negligible coloration in the absence of added peroxidatic catalyst. It remains generally true that users of chromogenic peroxidatic assays must formulate assay solutions from chromogen and hydroperoxide components stored separately, confronting the delay and wastage inherent in this procedure, as well as the need for careful mixing technique with clean equipment if they are to obtain good day-to-day precision. The dearth of peroxidatic indicator solutions showing storage stability for a practical period on the order of at least one year complicates the development and distribution of clinical diagnostic kits, especially for the home market, where a requirement that assay reagents be formulated at time of use decreases reliability and market appeal. Surprisingly, it has been found by the inventor that the more strenuous the efforts to remove transition metal ions and to sequester (by chelation) those metal ion contaminants which persist, the lower the degree of spontaneous color formation in solutions of hydroperoxides and chromogenic electron donors. Despite the extremely strong thermodynamic drive in favor of this background reaction, the truly uncatalyzed rate appears to be too low to measure on the time scale of months at temperatures between 0C and 40C, so that peroxidatic activity indicator solutions can be formulated with practical storage lifetimes well in excess of six months. Furthermore, it now appears that chelation is not a generic remedy to transition-metal-ion catalysis. Surprisingly, some chelators dramatically enhance the background reaction; others suppress it much more effectively than the standardly used strong chelator, EDTA; some chromogens are more effectively protected by chelators

than others; and the set of optimal chelators for one chromogen is not identical to that for another chromogen.

Summary

In a first aspect, the invention provides stable aqueous compositions for the visual, colorimetric, spectrophotometric or reflectometric indication of peroxidatic activity, comprising a buffer of pH between about 4 and 8, at least one chelator, a hydroperoxide, and a chromogenic electron donor of the benzidine family wherein the total concentration of oxidatively active transition metals is no greater than about 10^{-7} M, and wherein indicator solution storage lifetime in the dark in the temperature range of 0-40C is at least six months. The chelator preferably is taken from a select group shown to be especially effective in inhibiting oxidation of benzidine compounds.

A second aspect of the invention provides aqueous compositions for the colorimetric or spectrophotometric indication of peroxidatic activity, comprising a buffer of pH between about 4 and 8, a hydroperoxide, and o-phenylenediamine, wherein the total concentration of oxidatively active transition metals is no greater than 10^{-7} M, and wherein indicator solution storage lifetime in the dark is at least 2 weeks at 0-5C or 36 hours at 20-25C. The compositions optionally may contain certain chelating agents, such as DTPA and DFA.

A third aspect of the invention describes compositions comprising a chromogenic electron donor of the benzidine family in combination with at least one chelator taken from a select group shown to be even more effective than EDTA in inhibiting oxidation of benzidine compounds. The compositions may contain a hydroperoxide and an aqueous buffer of pH between 4 and 8, in which case they may serve to indicate peroxidatic activity. Without these additives, such compositions serve as stock solutions useful for preparing peroxidatic activity indicator solutions. The compositions also may contain an aqueous buffer of pH between 4 and 10 and a substrate for an oxidase enzyme. In this case, provided that they do not contain a hydroperoxide, they may serve to indicate activity of the oxidase.

In a fourth aspect, the invention provides methods of preparing peroxidatic activity indicator solutions with long storage lifetimes, involving combination of several measures effective for reducing transition metal contamination and preventing the photo-oxidization of chromogenic electron donors. In addition to a core group of essential practices, additional steps are taken as needed to meet storage-stability criteria in the face of the degree of transition-metal contamination of available component reagents.

A fifth aspect of the invention entails a method of storing peroxidatic assay indicator solutions which serves to promote long-term storage stability. It comprises the use of a plastic container shielded from light, which optionally may have been treated with a solution of a chelator or a strong mineral acid effective in reducing contamination with oxidatively active transition metals.

The invention includes assay kits which contain the claimed compositions and which benefit from the improved reliability and ease of use imparted by storage-stable peroxidatic activity indicator solutions.

Brief Description of the Figures

Figure 1 shows kinetic traces for TMB oxidation over five days' storage of TMB-H_2O_2 indicator solutions containing seven different chelators.

Figure 2 shows the kinetic traces for TMB oxidation over 125 days' storage of TMB-H_2O_2 indicator solutions buffered with succinate or citrate.

Figure 3 shows the effect of 10^{-3} M and 10^{-2} M EDTA on the ability of TMB-H_2O_2 indicator solution to support peroxidase catalysis over 125 days' storage.

Figure 4 shows the effect of pH on the background reaction of TMB-H_2O_2 indicator solution.

Figure 5 shows the effect of temperature on the background reaction of TMB-H_2O_2 indicator solution over the course of 13 days of storage.

Figure 6 shows the effect of preparation and incubation container composition on the background reaction of TMB-H_2O_2 indicator solution.

Figure 7 shows the effect oPD purity and chelators have on the background reaction of oPD-H_2O_2 indicator solutions at 25C.

Figure 8 shows the effect of pH on kinetics of the background reaction of oPD-H_2O_2 indicator solutions at 25C.

Description of the Preferred Embodiments

Advantage of the Invention

The present invention overcomes the difficulties in the prior art to provide ready-to-use aqueous indicator solutions for peroxidatic activity, containing both a hydroperoxide and a chromogenic electron donor, which remain visibly colorless and capable of sustaining full peroxidatic activity after storage at a wide range of temperatures for intervals of a day to many months. Such storage-stable liquid formulations of the substrates for peroxidatic reactions can be prepared economically in large batches and subjected to careful quality assurance before distribution, storage, and eventual use, with confidence that the ultimate user will obtain reproducible performance with less skill, equipment, effort, and delay than currently is the case. Because of the unusually wide storage temperature tolerance (between approximately 0° and 40°C) when a benzidine compound is used as chromogen, such formulations are especially suited for applications such as over-the-counter test kits and use in tropical environments, where refrigerated storage is impractical or very expensive.

Definitions

As used herein, "peroxidatic activity" refers to the ability of certain substances to accelerate the reaction of hydroperoxides with electron donors, especially colorless electron donors which become fluorescent or visibly colored after oxidation by an hydroperoxide.

"Peroxidatic assay" refers to any analytical procedure which relies on peroxidatic activity to create the signal which is detected or measured to infer the presence of analyte and/or the amount of analyte present. The two major classes of peroxidatic assay are (a) those in which a peroxidatic catalyst is the analyte and (b) peroxidase-linked specific binding assays.

"Chromogenic electron donor", or "chromogen", refers to a compound which undergoes an easily observed change in color upon oxidation by an oxidizing agent such as an hydroperoxide. Chromogenic electron donors are exemplified by four classes of substances: (1) the benzidine compounds, including benzidine, 3,3'-dimethylbenzidine (o-tolidine, oTD), 3,3'-dimethoxybenzidine (o-dianisidine, oDAD), 3,3'-diminobenzidine (DAB), 3,3',5,5'-tetramethylbenzidine (TMB), 3,3'-diethylbenzidine, and 2,7-diaminofluorene (DAF), (2) the phenylene diamines, including o-phenylenediamine (oPD), (3) 2,2'-azino-di(3-ethyl-benzthiazoline sulfonate (ABTS), and (4) aminoethyl carbazole (AEC).

"Hydroperoxide" refers to a compound of the general formula, ROOH, wherein the R group is an aryl, alkyl, or acyl group or an hydrogen atom. If R is an hydrogen atom, the hydroperoxide is known as hydrogen peroxide. If the R group is an aryl, alkyl, or acyl group, the compound is an "organic hydroperoxide". If R is the formula, $R_1R_2R_3C$ where R_1, R_2 and R_3 is any combination of aryl and alkyl groups other than hydrogen, the hydroperoxide is a tertiary alkyl hydroperoxide. If R_1 is a phenyl group and R_2 and R_3 are methyl groups, the hydroperoxide is cumene hydroperoxide. If R_1, R_2 and R_3 are methyl groups, the hydroperoxide is tertiary butyl hydroperoxide.

"Peroxidatic activity indicator solution" comprises a hydroperoxide in combination with an electron donor in liquid solution, which undergoes a measurable chemical or physical change when contacted with a peroxidatic catalyst. Preferred electron donors are chromogens or fluorogens. Preferred solvent is water, often in combination with buffer, a chelator, and/or an organic cosolvent.

A "chelating agent", or "chelator", is a compound which binds tightly to metal ions possessing at least two positive charges, by virtue of the fact that it carries at least two negative or electron-rich reactive groups which are directly bonded to the chelated metal ion. An "effective chelating agent" is one which, when added to an aqueous solution of an Fe(III) salt at a concentration no lower than that of Fe(III), lowers the peroxidatic activity of Fe(III) toward a given chromogen and H_2O_2 to a value no greater than approximately 1% of that of unchelated Fe(III). Different chromogens possess different sets of effective chelators. For chromogens which are benzidine compounds, examples

include ethylenediaminetetraacetic acid (EDTA), trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CTDA), ethylenediamine-N,N'-diacetic acid-N,N'-di-α-propionic acid (EDADP-α), ethylenediamine-N,N'-diacetic acid-N,N'-di-ß-propionic acid (EDADP-ß), ethylenediamine tetra-ß-propionic acid (EDTP-ß), ethylenediamine tetra-α-propionic acid (EDTP-α), N,N'-bis(2-hydroxybenzyl)ethylenediamine diacetic acid (HBED), ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDHA), desferrioxamine-B (DFA), and pyrophosphate (PP_i).

By "analyte-specific binding assay", or "specific binding assay", is meant an analytical procedure for detecting and/or quantitating a particular substance which depends on the binding to that substance of a second substance, known as a "binding moiety", which is not expected to bind to any other substance in the test sample or the test apparatus. The binding moiety is directly or indirectly linked to a signal-generating moiety, which is an enzyme in an "enzyme-linked analyte-specific binding assay". For the purpose of the present invention, the enzyme must be a peroxidase, preferably from a plant, most preferably from horseradish root. If the analyte is an antigen and the binding moiety is an antibody, or vice-versa, the enzyme-linked analyte-specific binding assay is known as an "enzyme immunoassay" (EIA) or an "enzyme-linked immunosorbent assay" (ELISA). If the analyte is an antigen or antibody attached to a cell or a tissue structure in a cytochemical smear or histochemical section and the binding moiety is an antibody or antigen specific for the analyte, the peroxidase linked analyte-specific binding assay is known as an "immunoperoxidase cytochemical or histochemical staining procedure". If the analyte is an antigen or antibody which has been captured on or in a manufactured solid support, the enzyme-generated signal is expected to stick to the solid support at the point of generation, and the binding moiety is an antibody or antigen specific for the analyte, the analyte-specific binding assay is known generally as an "immunoblot", or specifically as a "filter-trapped immunoassay", "immunodot blot", or "Western blot", depending on how capture occurs. If the analyte is a specific sequence of DNA or RNA bases and the binding moiety is a nucleic acid containing a base sequence complementary to at least part of the analyte sequence, the analyte-specific binding assay is known as a "nucleic-acid probe" procedure. If the nucleic acid analyte has been

captured on a manufactured solid support and the enzyme-generated signal is expected to stick to the support at the point of generation, the nucleic acid probe procedure is known as a nucleic-acid-probe dot blot or as a Southern or Northern blot, depending on how capture occurs and on whether the analyte is DNA or RNA. If the nucleic acid analyte is part of a cytochemical smear or a histochemical section, the nucleic-acid-probe procedure is known as an in situ nucleic acid hybridization assay.

"Test kit" refers to any combination of equipment, reagents, and/or instructions for the use of equipment and/or reagents to assay for the presence or amount of an analyte. Examples include test kits for performing peroxidase-linked analyte-specific binding assays such as EIA's, immunoperoxidase histochemical or cytochemical staining, and nucleic acid probe analyses, and for assaying for the presence of blood or hemoglobin in forensic or clinical test samples.

"Catalytic activity" of a peroxidatic catalyst is measured by the magnitude of the slope of the tangent to the kinetic trace observed when that catalyst is mixed with a chromogenic or fluorogenic electron donor and a hydroperoxide.

"Sensitivity" to a peroxidatic catalyst is a property of a peroxidatic activity indicator solution. It is just the catalytic activity seen when a specified amount of that catalyst is contacted with the indicator solution at a specified temperature. The amount of catalyst should lie within the range where catalytic activity is proportional to amount of catalyst.

"Storage lifetime" of a peroxidatic activity indicator solution is that interval after formulation of said solution during which (a) the absorbance of said solution in the 400-800 nm wavelength range (1cm light path) remains below about 0.03 and (b) the sensitivity of said solution to HRP does not range more than about 25% from its initial value.

"Oxidatively active transition metals" are those elements of Groups, IIIa, IVa, Va, VIa, VIIa, VIII and Ib of the Periodic Table which form ions which readily undergo 1-electron and 2-electron changes. Practically speaking, this group consists of Cr, Mn, Fe, Co, Ni, and Cr because only these elements, and especially Fe and Cr, are sufficiently common in chemical reagents to

catalyze a measurable reaction between hydroperoxides and chromogenic electron donors.

"Solution effective for dissolving transition metals" is an aqueous composition capable of oxidizing transition metals from their metallic (zero) oxidation state and/or dissolving any salts formed by their ionic (positive) oxidation states. Two classes of such compositions exist: dilute ($<10^{-2}$ M) solutions of strong chelators, such as EDTA, CDTA, DTPA and EDDHA, at pH values above approximately 4, and relatively concentrated (> 1M) solutions of strong mineral acids such as HNO_3, H_2SO_4, and HCl. Combination of these acids, such as $HNO_3 + H_2SO_4$, and HNO_3 + HCl, are more effective than each acid alone; and higher acid concentrations are more effective than lower concentrations. Mild heating (not exceeding approximately 100 C) improves the effectiveness of both classes of reagent. The time scale on which contacting surfaces with such reagents effectively dissolves all transition metals ranges from a few minutes to a few days, depending on reagent concentration, temperature and the nature of any metal contamination. "Metal-free" process means that the following two conditions are met:

(a) the substances subjected to the process do not contact any metal surfaces;

(b) all surfaces contacting the substances subjected to the process are plastic or have been contacted with a solution effective for dissolving transition metals. As applied to a liquid substance, "metal-free" means that the total concentration of oxidatively active transition metals is no greater than, and preferably much less than 10^{-7} M. For water in particular, "metal-free" implies that the water has been purified by at least one of the following processes:

(a) distillation in an apparatus, preferably made of glass or quartz, in which neither the liquid nor the vapor contacts metal;

(b) passage through a mixed-bed ion-exchange resin, an activated charcoal bed, and a filter with a nominal pore size below about 1 micrometer.

"Ion-exchange solid" is a solid material which is not dissolved by aqueous solvents and which contains chemical groups which ionize in aqueous solvents. These ionizable groups must be attached to the solid material in a

way such that they are not removed by aqueous solvents at mild pH values, between about 2 and 12, and at mild temperatures, between about 0 and 60C.

"Chelating solid" is a solid material which is not dissolved by aqueous solvents and which contains chemical groups which are chelators. These chelators must be attached to the solid material in a way such that they are not removed by aqueous solvents at mild pH values, between about 2 and 12, and at mild temperatures, between about 0 and 60C.

"Organic cosolvent" is an organic compound which does not react with water at mild pH values and temperatures and which is soluble in water to a concentration of at least about 5%. Preferred as organic cosolvents for this invention are N-methyl-2-pyrrolidone, ε-caprolactam, N-2-hydroxyethyl-2-pyrrolidone, N-ethyl-2-pyrrolidone, and N-cyclohexyl-2-pyrrolidone.

Modes for Carrying Out the Invention

The first aspect of the invention relates to aqueous peroxidatic activity indicator solutions in which the chromogenic electron donor is a benzidine compound, which contain a total concentration of dissolved and suspended oxidatively active transition metal not exceeding about 10^{-7} M, and which show practical storage stability of at least six months over the temperature range to which such solutions are commonly exposed during manufacture, storage, commercial transport, and use: 0-40C. Practical storage stability requires that these solutions remain visibly colorless and capable of sustaining approximately full peroxidatic activity after storage at a wide range of temperatures for intervals of a day to many months. In quantitative terms, these performance criteria mean that after storage for the specified period at a specified set of temperatures,

(a) the absorbance of the colored product of chromogen oxidation does not exceed a value of approximately 0.03 for a 1 cm light path in the visible wavelength region (400-800 nm) and;

(b) the assayed catalytic activity at a set temperature of a set concentration of the peroxidatic catalyst for which the indicator solution is designed does not change more than about 25% from its value with freshly formulated assay solution. To verify that a given indicator solution meets the functional storage stability standards of this invention, two simple measurements are done. After preparation of such a solution and initiation of storage in a

temperature-controlled environment, aliquots are withdrawn at recorded intervals (on the time scale of months) and immediately analyzed for visible spectrum and for ability to sustain peroxidatic activity. The spectrum is taken in a cuvette with a 1 cm light path after balancing the spectrophotometer with a solution containing all components of the indicator solution except the chromogen. For the purpose of the present invention, failure of the indicator solution absorbance at any visible wavelength to rise above approximately 0.03 units suffices to conclude that the baseline oxidation reaction has not introduced enough absorbance background to interfere with visual or instrumental monitoring of peroxidatic assay reactions. Ability to sustain peroxidatic activity is analyzed by adding a known and convenient concentration of a peroxidatic catalyst for which the indicator solution is effective and monitoring the increase in absorbance at the wavelength maximum of the oxidized chromogen at a measured (and preferably controlled) temperature on the time scale of one to ten minutes. In this context, "convenient" means a catalyst concentration giving an easily measured absorbance change, preferably between 0.1 and 2, in five to ten minutes. The peroxidatic activity can be defined as either the initial slope of the graph of absorbance versus time or as the total absorbance change in a set time interval after addition of the catalyst to the indicator solution. When divided by the catalyst concentration in the cuvette, the peroxidatic activity at a set temperature should remain approximately constant over the storage lifetime of the indicator solution, not changing more than about 25% from its value for freshly prepared indicator solution. Peroxidatic activity indicator solutions which meet these storage stability criteria should also show reproducible performance in very different assay formats, such as arise in various immunoassays, nucleic acid probe assays, tests for the presence of blood in clinical and forensic specimens, and histochemical and cytochemical procedures. The specific details of these functional tests must be optimized for the indicator solution being tested, in ways understood by one skilled in the art of enzymology. For example, the blue product of TMB oxidation has a λ_{max} at 652 nm, whereas the analogous value for DAF oxidation is 602 nm. At pH 5, DAF is approximately ten times more soluble than TMB in the absence of cosolvent; but its sensitivity to HRP is about 10% of that of TMB, so that a higher concentration of HRP in the assay cuvette is needed to give DAF

oxidation rates comparable to those of optimized TMB-containing indicator solutions. These HRP concentrations are on the order of 10^{-11}-10^{-10} M.

Peroxidatic activity indicator solutions contain minimally the following three components: a chromogenic electron donor, a hydroperoxide, and a buffer. Optionally an organic cosolvent is included to increase chromogen solubility. The chromogens covered by the first aspect of the invention are in the benzidine family of compounds. Commercially available members of this family include benzidine, o-tolidine, 3,3'-diethylbenzidine, 3,3',5,5'-tetramethylbenzidine, o-dianisidine, and 2,7-diaminofluorene. Although all can be protected against transition-metal-ion-catalyzed oxidation in the compositions and by the methods of this invention, TMB is greatly preferred over all of the others by virtue of its greater sensitivity to HRP and its established safety. Those benzidine compounds (including DAF) with hydrogen atoms at any of the substitution positions ortho to the amines (3,3',5, or 5' positions of benzidine) are all cancer-suspect agents; some are verified carcinogens or mutagens.

Commercially available hydroperoxides include hydrogen peroxide, (H_2O_2), urea hydrogen peroxide (a solid form of H_2O_2 which becomes functionally equivalent to H_2O_2 once it is dissolved in an aqueous solvent), and the tertiary alkyl hydroperoxides: t-butyl hydroperoxide and cumene hydroperoxide. Various other alkyl hydroperoxides, such as methyl hydroperoxide and ethyl hydroperoxide, and peroxyacids, such as peroxyacetic acid, are well described in the research literature but lack the chemical inertia required for safe commercial production and distribution. Preferred oxidants in indicator solutions are hydrogen peroxide and urea hydrogen peroxide because of their unlimited aqueous solubility and relative non-toxicity. Additionally, they normally are much more sensitive to peroxidatic catalysts than the alkyl hydroperoxides or peroxyacids.

Although various peroxidatic catalysts can be beneficially assayed over a broad pH range, on the order of pH 3 to pH 10, most such assays, especially of plant peroxidases such as HRP, are performed optimally in the pH 4-6 range. For example, HRP is maximally active with TMB and H_2O_2 at pH values near 4. Furthermore, the rate of transition-metal-ion-catalyzed dismutation of hydroperoxides often is proportional to hydroxide ion concentration, so that low pH values are preferred for peroxidatic activity

indicator storage stability. Preferred buffer species in the 4-6 pH range include citrate, succinate, piperazine, N-methyl piperazine, benzoate, o-anisate, fumarate, pyrophosphate and pyromellitate. Piperazine and N-methyl piperazine are preferred because they can be sublimed or distilled under metal-free conditions to minimize metal-ion contamination. On the other hand, they are preferred only for peroxidatic activity assays in which the chromophoric oxidation product is soluble or forms an insoluble polymer. If a benzidine compound is used under conditions where the colored oxidation product is expected to be immobilized by binding to a polymeric anion or precipitation as an insoluble salt, these two amine buffers are not preferred because they compete with the cationic oxidation product for the immobilizing polymeric anion or precipitating anion. [This analytically useful mode of trapping the colored product of benzidine oxidation is described in copending U.S. Patent Application 896,677, filed August 20, 1986, disclosure of which is incorporated herein by reference.] Citrate is preferred as a buffer species because it is commercially available with very low levels of metal ion contamination; it does not serve well as a product-precipitating anion, but it also does not interfere with ionic trapping of the colored oxidation product. Benzoate and o-anisate are preferred buffers because their acids are easily recrystallized by dissolving in hot water in the presence of a strong chelator and chilling the solution. This procedure is effective in removing trace transition-metal-ion contamination. They too do not interfere with and do not effectively promote ionic trapping of the colored oxidation product. Fumarate and pyromellitate (1,2,4,5-benzene tetracarboxylate) are preferred when it is desired to precipitate the colored oxidation product as a salt; and the latter is easily recrystallize as described above, i.e., by dissolving in hot water in the presence of a strong chelator and chilling the solution. It may be beneficial to formulate a buffer as a mixture of two or more of these preferred buffer species, for example, a small amount of a precipitant like pyromellitate and a larger amount of a cheap and very pure, but non-precipitating, buffer such as citrate. However, the preceding description of useful buffer species is in no way exclusive. The general functional criteria for an indicator solution buffer species is that it control pH adequately and that it be available in a form substantially free of contaminating transition metal ions or else be easily repurified to exclude such impurities.

The concentrations of chromogen, hydroperoxide, and buffer in the indicator solution are set by several functional criteria, having to do with sensitivity to peroxidatic catalysts as well as with storage stability. The various benzidine chromogens have individual solubilities which are pH dependent and which must not be exceeded in a storage-stable formulation; their concentrations in storage-stable indicators must be below the solubility at every temperature in the specified stability temperature range. Practically, their concentrations should not exceed their solubilities at OC. However, within that limit, the highest concentration normally is desirable to give the greatest sensitivity toward peroxidatic catalysts. For example, TMB is normally used at a concentration of 0.3-0.4 mM at pH 4.0 without organic cosolvent, 0.06 - 0.1 mM at pH 5.0 without organic cosolvent, and 0.3-0.4 mM at pH 5.0 with 10% N-methylpyrrolidone as organic cosolvent. On the other hand, if the resulting sensitivity exceeds the needs of the assay for which the indicator solution is intended, a lower chromogen concentration gives greater protection against exceeding the storage stability absorbance limit (0.03 absorbance units), as the background reaction rate varies directly with chromogen concentration.

Organic cosolvents vary widely in ability to increase TMB solubility, but also generally reduce HRP activity, so that their net effect on indicator solution sensitivity with chromogen concentration near its solubility limit often is marginal. Preferred among organic cosolvents for increasing TMB solubility with minimal reduction of HRP activity is N-methylpyrrolidone at a concentration of 5-10 weight percent. However, such concentrations may render the organic cosolvent the major contributor of transition-metal-ion impurities to the indicator solution, unless the cosolvent is rigorously repurified. Therefore, it is preferred to formulate storage-stable peroxidatic indicator solutions without organic cosolvent in order to avoid the associated process costs. Furthermore, whatever chromogen solubility (and therefore indicator sensitivity) benefits derive from organic cosolvent use are seen only at pH values above about 4.5, where the benzidines generally carry no molecular charge [the exact limit depends on the value of the second pKa of the individual benzidine compound]. At lower pH values, where benzidine protonation increases solubility, organic cosolvents become even more marginally beneficial because their effectiveness in increasing chromogen solubility is reduced.

Hydroperoxide concentration in the indicator solution normally is set to maximize sensitivity toward the peroxidatic catalyst to be assayed, as there are no practical solubility limits for most of the hydroperoxides. [An exception is cumene hydroperoxide, with an aqueous solubility near 20 mM in the absence of detergent or organic cosolvent.] For example, HRP activity with TMB is maximal when the H_2O_2 concentration is 1-3 mM, declining gradually at higher concentrations and sharply as the H_2O_2 concentration drops below 1 mM. However, if maximum sensitivity is not required for the intended use of the indicator solution, lowering the hydroperoxide concentration is beneficial for two reasons.

(a) Lower hydroperoxide concentrations give lower rates of background absorbance increase, simplifying the task of meeting the indicator solution absorbance stability goal.

(b) The oxidation mechanism of benzidine compounds consumes two molecules of benzidine per molecule of the blue oxidation product, which is a 1:1 charge-transfer complex between a yellow 2-electron oxidation product and colorless TMB. If the test sample to which the indicator solution is exposed is particularly rich in peroxidatic catalyst and if the hydroperoxide molar concentration exceeds half the benzidine concentration, the initially formed blue color may turn to yellow when the catalyst consumes all the free benzidine and starts to oxidize the benzidine contained in the charge-transfer complex. There are several ways to avoid such an effect, which does not influence assay detection limit but reduces the dynamic range. One way is to reduce the hydroperoxide concentration. There is no further benefit from this remedy once the hydroperoxide has been lowered to half the benzidine compound concentration.

Preferably the lowest indicator solution buffer concentration is used which gives effective pH control in the assay of peroxidatic activity, normally a concentration in the range of 1-10 mM. This precaution helps to minimize transition metal ion contamination because buffer components often are major sources of impurities. Buffer concentrations above 10 mM are not incompatible with the invention but complicate its realization.

The first aspect of the invention includes a compositional criterion: minimal contamination with oxidatively active transition metals. Practically speaking, this group consists of Cr, Mn, Fe, Co, Ni, and Cu. The 10^{-7} M contamination threshold is consistent with attainment of the storage-stability criterion of this aspect of the invention. Verification that a given indicator solution meets the compositional standards of this invention (with respect to transition-metal-ion contamination) can be done by any of several methods known in the field of instrumental analytical chemistry, including Inductively Coupled Plasma (ICP) Emission Spectroscopy, atomic absorption (AA) spectroscopy, and ion chromatography. Preferably the solution will be evaporatively concentrated before analysis; reducing the volume 90-99% can increase sensitivity 10-100 fold. Also, preferably a multi-element analysis protocol will be used, so that all of the potentially relevant transition metals can be measured in a single sample. However, single-element analyses by graphite furnace AA spectroscopy are the most sensitive commonly available methods, and may be used without evaporative concentration. It is essential that the most common contaminants, Fe and Cr, be measured and is highly preferred to track Mn, Co, Ni, and Cu. Furthermore, it is preferred to monitor the transition metal contamination of the bulk chemical components used to make up the indicator solutions, because it may often be easier to estimate a contaminant at the source than in the final formulation, where it is more dilute.

One of the most effective ways to realize the invention is to include one or more chelators in the indicator solution. However, all chelators are not equally effective in suppressing the background reaction of peroxidatic activity indicator solutions; and some actively promote the reaction. Furthermore, not all chromogens are benefitted equally by chelation, and the optimal chelators for one chromogen may not all be preferred for another chromogen. Preferred chelators for chromogens taken from the benzidine family are desferrioxamine B (DFA), bis(hydroxybenzyl)ethylenediamine (HBED), ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDHA), ethylenediaminetetra-α-propionic acid (EDTP-α), ethylenediaminetetra-ß-propionic acid (EDTP-ß), ethylenediamine-N,N'-diacetic-N,N'-di-α-propionic acid (EDADP-α), ethylenediamine-N,N'-diacetic-N,N'-di-ß-propionic acid (EDADP-ß), pyrophosphate (PP_i), 1,2-trans-

cyclohexanediaminetetraacetic acid (CDTA), and ethylenediaminetetraacetic acid (EDTA). DFA is supplied by CIBA-GEIGY under the tradename, Desferal. HBED may be prepared by the method of L'Eplattenier et al. [(1967) J. Amer. Chem. Soc., 89:837-843], the disclosure of which is incorporated herein by reference. EDTP-α and EDTP-ß may be prepared by a variation of the standard procedure for making EDTA: mild heating of ethylenediamine with α-chloropropionic acid or ß-chloropropionic acid in an aqueous solution at high pH, followed by acidification with strong mineral acid to precipitate the chelator as the free acid. The other chelators listed are commercially available as research reagents. Commercial EDDHA is normally so impure with metal ions that it should be repurified one or more times by the procedure of Rogers [(1973) Infection and Immunity, 7:445-456], the disclosure of which is incorporated herein by reference.

Effective chelator concentrations range from 10^{-5} M to 10^{-2} M, with 10^{-4} M to 10^{-3} M preferred as a compromise between two trends. Higher chelator concentrations may help to compete with other metal-ion ligands (e.g., citrate or succinate buffer species) which form complex ions catalytically more active than the preferred transition-metal-ion chelates. However, commercially supplied chelators often are major sources of transition-metal-ion contamination, so that increasing chelator concentration beyond the level needed to sequester metal ions introduced with other indicator solution components may reduce storage stability. Therefore lower chelator concentrations may be more stabilizing. The optimal choices of chelator identity and chelator concentration may vary with the choice of chromogen and the purity of the individual components of the indicator solution. Addition of several chelators may be beneficial to broaden the effectiveness of the invention in suppressing transition metal-ion catalysis.

Sometimes the concentration of a benzidine compound in an indicator solution will be observed to decline by 5-20% during storage over weeks to months, without any indication of oxidation (color formation). This loss, due to adsorption to the walls of the plastic storage vessel, can be reduced by pre-treatment of the vessel with a preparation which renders the walls hydrophilic, such as is supplied by Seton Scientific (Sunnyvale, CA).

In a second aspect, the present invention offers storage-stable aqueous peroxidatic activity indicator solutions in which the chromogenic electron donor is o-phenylenediamine (oPD). Practical storage stability is operationally defined for oPD as for chromogens of the benzidine family of compounds, and the wavelength region for monitoring storage stability is 440-450 nm. However, because oPD is sufficiently more reactive than the benzidines, the present invention assures stability for only approximately 36 hours at 25C or two weeks at 5C. Preferably the hydroperoxide of the indicator solution is hydrogen peroxide at a concentration between about 2 mM and about 6 mM, and the buffer pH is between about 4 and about 6. Storage stability is increased several-fold by lowering the pH from 5.5 to 4.5, and pH 4.5 is also optimal for sensitivity to HRP. Preferred buffer species are citrate, piperazine, and N-methylpiperazine, used separately or together, at concentration(s) between about 10^{-3} and 10^{-1} M, preferably about 10^{-2} M. Chelation is of marginal value for stabilizing oPD indicator formulations, but DTPA and DFA may improve performance over that of citrate alone. Preferred chelator concentration is in the range of 10^{-3}-10^{-2} M. DTPA and citrate can provide anions to match the cation provided by piperazine or N-methyl piperazine, so that especially preferred buffers contain one of these two bases in combination with enough citric acid or DTPA free acid to provide the desired pH, optimally near pH 4.5. Choice of oPD concentration is focal to achieving the targeted storage stability. oPD solubility greatly exceeds the oPD Michaelis Constant (Km) of HRP (the catalyst for which oPD is most often used as chromogen). As sensitivity to HRP increases only marginally as oPD concentration rises above its Km, and the background reaction rate increases in proportion to the oPD concentration, storage stability is greatly promoted at little cost to assay sensitivity by keeping the oPD concentration in the vicinity of the Km (3 mM at pH 4-5).

In a third aspect, the invention specifies compositions which protect benzidine compounds from oxidation by combining with them effective amounts of one or more of those chelators found to be substantially more protective than EDTA, currently standard in the art. These chelators comprise DFA, HBED, EDDHA, EDTP-α, EDTP-ß, EDADP-α, EDADP-ß, CDTA, and pyrophosphate. In its most general embodiment, this aspect employs any useful

liquid as solvent, with N-methylpyrrolidone preferred for chromogen stock solutions and aqueous buffers preferred for indicator solutions, as noted above; and it does not require the presence of hydroperoxide. It includes compositions wherein a solution of specified chromogen and specified chelator, with or without a hydroperoxide, is imbibed into a porous solid, such as a paper, and allowed to dry to form a test strip. However, in another embodiment, this aspect of the invention is realized by peroxidatic activity indicator solutions containing these chromogens together with the preferred chelators, independently of whether the functional or compositional criteria of the first aspect of the invention are met. The modes for constructing such indicator solutions are those described above, except that there is no requirement for any specific procedural step of the fourth aspect of the invention, described below, beyond inclusion of one of the listed chelators. In a third embodiment, this aspect of the invention relates to indicator solutions for oxidase activity. Such solutions comprise a benzidine compound in combination with a preferred chelator, an aqueous buffer, and an electron donor, which is a preferred substrate for the targeted oxidase enzyme. For example, glucose is added to detect glucose oxidase. As understood by one skilled in the art of enzyme-linked assays, such an indicator solution is used by contacting it with a test sample in combination with a peroxidase enzyme, such as HRP. If the test sample contains the targeted oxidase, it will catalyze reduction of dissolved oxygen to hydrogen peroxide by the oxidase substrate. This hydrogen peroxide then will oxidize the benzidine chromogen under catalysis by the added peroxidase. The benzidine compound may be supplied as the salt of a strong mineral acid, such as the commercially available di-HCl salts of TMB and oDAD, even though such salts commonly are less pure than the free bases. The chelators may be supplied as the neutral compounds or sodium salts, as convenience or purity dictates.

Because the transition metal ions act catalytically, and because even metal ion chelates generally retain some catalytic activity, storage lifetime is expected to vary in inverse relationship to the residual transition metal ion concentration. Accordingly, there is almost no limit to the practical benefit from improved procedures to eliminate trace transition metals from peroxidatic activity indicators. Therefore in a fourth aspect, the invention provides a set

of procedures which individually do not suffice to meet the functional and compositional criteria of the first two aspects, but which as a group effectively attain these ends. Outside of an essential core of practices, additional steps may be taken as dictated by the individual formulation conditions, such as the level of contamination of the available indicator solution components.

The essential steps to be taken in preparing storage stable and substantially metal-free indicator solutions are the following:

(a) preparation and especially storage of the final composition and all intermediate mixtures on the pathway to making the final composition (1) in plastic containers or containers which have been chemically treated to dissolve any contaminating oxidatively active transition metal and (2) without contact with metal;

(b) use of solvent water which has been distilled in glass or quartz or (preferably) purified by a filtration system comprising a mixed-bed ion-exchange resin, an activated charcoal filter, and finally a sub-micrometer particle filter;

(c) storage shielded from ultraviolet light and, in the case of oPD indicator solutions, formulation without direct illumination with light (e.g., fluorescent light) containing a significant ultraviolet component.

The first precaution recognizes that not only stainless steel, but also glass, surfaces contain or can trap by adsorption a substantial amount of oxidized transition metal, principally iron, which can be dissolved in aqueous solvents contacting them, especially solvents of relatively low pH or containing chelators (including citrate). Containers and other equipment used for indicator solution preparation and/or storage preferably are incubated with chelator solutions or any of the standard mineral acid formulations used to clean equipment for trace element analysis. This dissolution precedure is required of magnetic stir bars, which tend to collect visible and microscopic iron filings, strongly preferred for glass, and still advisable for plastic. Plastic containers, stirrers, filters, and other equipment contacting the indicator solution and its components during manufacture may be composed of practically any polymer. However, storage preferably is in virgin unpigmented polyethylene or polypropylene, because the other most economical choice, polystyrene, is likely to adsorb the chromogens from solution, lowering chromogen concentration and

indicator sensitivity to peroxidatic catalysts. The second precaution recognizes that water, as the majority component of indicator solutions, must meet the highest purity standards. The water purification system, whether using distillation or ion exchange to remove metal ions, should not contact the purified water with any metal surfaces, and the purified water should not be stored in glass. Many commercially available deionization systems, provided by such companies as Millipore Corporation, Vanguard International, Inc., Barnstead Company, Culligan, and Continental Water Systems Corporation, meet the standards of the invention. Preferably the water will be subjected to at least two passes through purification systems of the specified kinds. Dark storage of chromogen solutions, in the presence or absence of a hydroperoxide, is essential to avoid photo-oxidation of these aromatic molecules, which rapidly violates the absorbance storage stability criterion. Chromogens are most sensitive to ultraviolet light (below approximately 400 nm), but the preferred protective tactic is to avoid all light. A foil or other opaque shield around a colorless plastic storage container is preferred to using an opaque plastic, as plastic pigmentation may introduce metal ions. oPD is more sensitive that the benzidine compounds to photo-oxidation, and so should be protected from at least ultraviolet light during the few minutes required for dissolution and formulation.

In addition to these three minimal procedural steps, other preferred operations for meeting the functional storage stability criteria include the following, which may be taken separately or together.

(a) The completely formulated indicator solution is filtered before storage. The final microfiltration, which can be performed with any of many commercially available plastic devices from companies such as Gelman Sciences, Millipore Corporation, and Nuclepore Corporation, removes dust and colloidal transition metal hydroxide particles and, at the 0.2 micrometer level, excludes microorganisms which contain oxidatively active transition metals and also may be able to metabolize buffer components. A novel microporous alumina membrane produced by Anotec Separations may provide improved filtration over the accepted state of the art, having nominal pore sizes down to 0.02 micrometer.

(b) Chromogen and/or buffer components are repurified by distillation or sublimation, or by recrystallization in the presence of a chelator. Repurification by sublimation is preferred for piperazine, oPD, and the benzidine compounds. As free bases, they are crystalline solids with melting points between 100 and 200C. Sublimation, effectively performed at a temperature 3-10C below the melting point in a vacuum of 0.1-10 mm Hg, is preferred because the compound does not pass through a liquid phase wherein it can take up metal ion impurities from the walls of the container. Benzoic acid, o-anisic acid, and pyromellitic acid, like aromatic acids in general, show large changes in aqueous solubility with temperature; recrystallization from water purified as described above, in the presence of 10^{-4}-10^{-2} M of a strong chelator, such as EDTA or DTPA, which will not coprecipitate with the aromatic acid, is the preferred repurification method for such compounds. Citric acid also can be repurified in this manner, despite the fact that it is much more water-soluble than the aromatic acids.

(c) If the chromogen is a benzidine compound, a chelator, taken from the group consisting of DFA, EDDHA, HBED, EDTP-α, EDTP-ß, EDADP-α, EDADP-ß, CDTA, EDTA, and pyrophosphate, is added to a concentration between about 10^{-5} M and 10^{-2} M, preferably between about 10^{-4} M and 10^{-3} M.

(d) If the chromogen is oPD, a chelator taken from the group consisting of DFA, DTPA, and citrate (often used as a buffer anyway) is added to a concentration between about 10^{-4} and 10^{-2} M.

(e) If a chelator is used, it is synthesized or repurified under metal-free conditions. For example, some of the specified chelators, such as EDTP-α, EDTP-ß, EDADP-α, EDADP-ß, DTPA and CDTA, resemble EDTA closely enough in structure that their sodium salts can be precipitated from ethanol/water essentially as described for Na_2 EDTA by Blaedel and Knight [(1954) Analytical Chemistry, 26:741-743], the disclosure of which is incorporated herein by reference. For this procedure to be maximally effective, the aqueous chelator solution is incubated, for an interval of at least about 10 minutes before ethanol addition, with an effective amount of a stronger chelator, such as DFA, HBED, or EDDHA, which is not precipitated by ethanol. Furthermore, this procedure requires ethanol which is particularly free of transition-metal-ion

contamination. An alternative repurification procedure for the free-acid forms of the same family of chelators is described by Zief and Mitchell [(1976) Contamination Control in Trace Element Analysis, Wiley-Interscience, New York, pp. 129-130], the disclosure of which is incorporated herein by reference. This method has the advantage of avoiding concern about ethanol contamination. Metal-free chelator synthesis is done by any of the standard published synthetic procedures, excepting that none of the reactants or products contacts metal, that glass equipment is avoided in favor of plastic whenever possible, that the apparatus is cleaned in advance with aqueous chelator solutions or mineral acid formulations commonly used in the art of trace element analysis [Zief and Mitchell, supra, pp. 92-95, the disclosure of which is incorporated herein by reference], and that the reactants are repurified (e.g., by distillation, sublimation, or recrystallization) before use if necessary to reduce the transition metal ion load which they contribute to the final product. Effective execution of this procedure requires elemental analysis for transition metals by methods such as those described above, both of synthetic reagents and of the commercially supplied chelator, to evaluate what must be done to render metal-free synthesis or repurification an improvement.

(f) Alternative commercial supplies of indicator solution components are subjected to trace metal analysis as described above, followed by choice of those materials which are least contaminated with oxidatively active transition metals.

(g) Soluble trace transition metal ions are removed from indicator solutions, liquid components of indicator solutions, or intermediate mixtures created during indicator solution preparation, by contact with a solid ion-exchange or chelating material. The major limitation on this method is that it must be done at a preparative stage before soluble chelator is added. Preferably the ion-exchange or chelating solid is packed in a column so that the liquid is flowed over it in a continuous mode, with flow rate limited to a value which gives complete extraction of metal ion from the liquid. Preferred solids for metal ion extraction are Amberlite 200, Amberlyst 15, Amberlite IRC-718 [Amberlite and Amberlyst are tradenames of Rohm and Haas Company], Duolite C264, Duolite ES346, Duolite ES466, Duolite ES467 [Duolite is a tradename of Chemical Process Company], Chelite C, Chelite P, Chelite N

[Chelite is a tradename of SERVA Fine Biochemicals], activated basic alumina, activated silica, poly(acrylamidoxime)resin [Colella et al. (1980) Analytical Chemistry, 52:967-972, the disclosure of which is incorporated herein by reference], and anion exchange resins loaded with anionic aromatic complexing agents such as Thoron [Brajter et al. (1985), Mikrochimica Acta [Wien] (1985), 179-186, the disclosure of which is incorporated herein by reference]. Macroreticular ion exchange and chelating resins are preferred over gel-type resins, such as Chelex-100 (Dow Chemical Company), because they are mechanically more durable than gel-type resins and are less likely to leach soluble polymeric fragments into the effluent. In the case of Chelex 100, such soluble polymers retain their chelating ability and actually activate transition metal ions as oxidative catalysts. Chelating solids also may be added to the indicator solution during storage to scavenge trace transition metal ion contaminants.

All compositional aspects of the invention comprise not only the described compositions, but also test kits which include or recommend the use of such compositions in order to simplify or improve the reliability of assays of peroxidatic or oxidative activity. Such a kit may be used in practically any analytical format, including ELISA, rapid filter-binding enzyme immuno-assay, Western blot, immunodot blot, Southern blot, Northern blot, nucleic acid hybridization dot blot, occult blood testing, forensic blood testing, and peroxidase-linked histochemical and cytochemical staining procedures. Because the oPD-containing compositions of this invention do not have the long-term storage-stability properties necessary for distribution in manufactured form, they are most likely to be realized in test kits as separate containers of rigorously purified solid oPD and a hydroperoxide-containing buffer solution, which upon mixing by the user yield an indicator solution with the specified storage stability. On the other hand, kits using storage-stable indicators which contain a benzidine chromogen supply such indicator solutions in ready-to-use form.

The uses of the indicator solutions of this invention are common knowledge among those skilled in the arts of analytical, clinical, and forensic chemistry, and in no way limit the compositions and processes described.

Example I

Effects of Chelators in the Fe(III)-Catalyzed Peroxide Oxidation of Benzidine Compounds: 5 Minute Assays

The following sublimations were performed at about 1 mm Hg to repurify commercially supplied reagents: piperazine (Sigma Chemical Co.) at 80-90C, benzidine (BZ, Sigma Chemical Co.) at 123-128C, 2,7-diaminofluorene (DAF, Aldrich Chemical Co.) at 158-163C, o-dianisidine (oDAD, Tokyo Kasei Co.) at 130-136C, and o-tolidine (oTD, Sigma Chemical Co.) at 124-128C. 3,3',5,5'-tetramethylbenzidine (TMB, Aldrich Chemical Co.) was so pure that sublimation was unnecessary. All benzidine compounds were dissolved in N-methylpyrrolidone (Burdick and Jackson) in polypropylene tubes to make 0.1 M or 0.02 M stock solutions and stored at -18C. Peroxidatic activity indicator solutions were made immediately before measuring the catalytic activity of Fe(III) preparations by mixing 10 µl of a stock solution of a benzidine compound with 500 µl of a 3 mM solution of H_2O_2 in 0.1 M piperazine HCl, pH 4.0, the latter solution being formulated fresh daily. The final indicator solution concentration of all benzidine compounds but TMB was 2.0 mM; TMB was used at 0.4 mM. Stock 0.5 M $FeCl_3$ was mixed with water and 10^{-1} M chelator stock solutions, pH 4-6, to prepare catalyst stock solutions which were 5 mM in $FeCl_3$ and 50mM in chelator. The following chelators were processed in this manner: hydroxyethylenediaminetriacetic acid (HEDTA), nitrilotriacetic acid (NTA), 3,3',3" -nitrilotripropionic acid (NTP), N,N'-ethylenediaminediacetic acid (EDDA), ethylene glycol-bis(ß-aminoethylether)N,N,N',N'-tetraacetic acid (EGTA), N,N-dihydroxyethylglycine (DHG), sodium pyrophosphate (PP_i), oxalic acid (OX), citric acid (CIT), EDTA, DTPA, TTHA, CDTA, EDADP-α, EDADP-ß, and DFA. One chelator, EDDHA, was kept in an 0.05 M stock solution at pH 8.4, and was mixed with 0.5 M $FeCl_3$ without added water to make a catalyst stock solution which was 5.0 mM in Fe(III) and slightly less than 50 mM in EDDHA. The DFA and EDDHA chelator stock solutions were stored at 5C, the latter covered with aluminum foil; the other chelator stocks were stored at room temperature. Because acidification by $FeCl_3$ reduced EDDHA solubility, this stock solution had to be prepared immediately before use, as EDDHA precipitated from it after standing several hours at room temperature. All chelators except DFA and EDDHA were reagent-grade material, supplied by

Sigma Chemical Co. or Mallinckrodt, Inc. or in one case (DHG) Eastern Chemical Co. EDDHA, commercially supplied (Aldrich Chemical Co.) as a tan solid which gave at least 6 peaks by high-pressure hydrophobic interaction chromatography (HPHIC) on a phenyl-TSK column (Toyo Soda Co), was repurified by a variant of the procedure of Rogers, supra, differing from the published method in that (a) the EDDHA was dissolved without boiling, adding concentrated HCl dropwise to obtain complete dissolution; (b) 10^{-3} M DTPA was added before neutralization; and (c) one volume equivalent of acetone was added instead of 7.5 equivalents. Repurified EDDHA showed only three trace impurities by HPHIC monitored at 300 and 450 nm. DFA, supplied by CIBA-GEIGY under the Registered Trademark, Desferal, was used without further purification.

The peroxidatic activities of these chelated Fe(III) preparations and unchelated $FeCl_3$ were measured by diluting stock catalyst 1/51 into freshly mixed indicator solution in a disposable polystyrene spectrophotometer cuvette (1 cm light path) in a Hewlett-Packard HP8450A spectrophotometer, recording for five minutes the absorbance at the λ_{max} for the colored oxidation product of a given benzidine compound (these wavelengths are reported in Table I). This dilution gave final concentrations in the cuvette of 10^{-4} M for Fe(III) and 10^{-3} M for chelator. Some Fe chelators and unchelated $FeCl_3$ were so active that the stock catalyst solutions were diluted 1/10 or 1/50 in deionized H_2O immediately before assay, giving final concentration of 10^{-5} M or 2×10^{-6}M. Failure to assay $FeCl_3$ immediately after dilution resulted in a substantially reduced rate, presumably because of hydrolysis and $Fe(OH)_3$ aggregation. The rates in Table I, reported as the total absorbance change over five minutes, were all corrected to an Fe(III) concentration of 10^{-4} M assuming that rate is proportional to catalyst concentration. Before this dilution correction, measured rates were corrected for any baseline oxidation rate caused by endogenous catalyst in the indicator solution or adsorbed to the cuvette walls, measured by recording absorbance for five minutes before adding prepared catalyst. Baseline rate, rechecked often during a series of assays, ranged from 10^{-3} to 10^{-2} absorbance units per five minutes and showed a precision of $\pm$ 0.003 absorbance units per five minutes for a given benzidine compound and cuvette.

Table I summarizes the dilution corrected, baseline-corrected rates for a series of catalysts acting on a series of benzidine compounds, most values being averages of at least two rate measurements. The reaction temperature was 24-26C. The table suggests the following conclusions.

(1) Chelators have no generic value in suppressing transition-metal-ion catalytic activity, contrary to the claims of European Patent Application No. 123,902, incorporated herein by reference. For chromogens such as DAF and TMB, Fe(III) catalytic activity ranges over 5 to 6 orders of magnitude, depending on the chelator chosen. Two chelators, DHG and EDDA, actually activate Fe(III) relative to unchelated metal ion. Consequently, the ability of some chelators to be especially effective protectants of benzidine compounds could not be predicted from any general theory of or experience with chelation.

(2) Although there is a rough trend in activating ability, chelators do not observe a strict rank order with respect to ability to protect benzidine compounds. For example, EDTA is twice as effective as citrate in protecting TMB but only a third as effective as citrate in protecting oDAD. DTPA, considered by many workers to be a "super-chelator" comparable to DFA, is no better than EDTA in protecting oDAD and DAF and half as good as EDTA in protecting TMB; it is worse than citrate in protecting TMB and oDAD. Consequently, the ability of some chelators to be especially effective protectants of some benzidine compounds could not be predicted from their behavior in other systems or with other benzidine compounds.

(3) Benzidine compounds vary significantly in vulnerability to Fe(III)-catalyzed H_2O_2 oxidation, which tends to parallel sensitivity to HRP catalysis. DAF and TMB, the chromogens which are most sensitive to HRP, are also the most useful in discriminating among chelators. The fact that a much larger number of chelators completely eradicate Fe(III) activity on BZ and oTD than on oDAD, DAF, or TMB reports only on the relative insensitivity of BZ and oTD toward all oxidation catalysts. It does not show that all of these chelators are equally protective of BZ and oTD.

(4) Five chelators, DFA, EDDHA, PP_i, EDADP-α, and CDTA, are significantly better protectants of benzidine compounds than citrate and EDTA, currently standard in the field; in most cases they improve stability by more

than an order of magnitude. A sixth, EDADP-ß, surpasses EDTA with oDAD and matches EDTA with the other chromogens.

This experiment has used an unrealistically high Fe(III) concentration in order to screen efficiently, in a short time period, a large number of chelators on several chromogens. Because catalytic activity normally is proportional to catalyst concentration, the discovery of five chelators with unprecedented protective power is expected to generalize to the much lower transition metal ion concentrations and longer incubation periods relevant to indicator solution storage stability, where the metal ion is an accidental contaminant rather than an introduced component. The use of such high Fe(III) concentration also renders unlikely the possibility that Fe(III) contamination of the chelator itself artifactually influences apparent chelator effectiveness, a potential complication of direct comparison of chelator effects on long-term storage ability. Of course, Fe is only one of several possible contaminating transition metals in real life. However, elemental analyses in Example 3 show that it is practically the only significant contaminant in most chelator and buffer components, so that effective control of Fe(III) catalytic activity should suffice in most cases to protect indicator solutions.

TABLE I

Effect of Chelators on the Peroxidatic Activity of 10^{-4} M Fe(III) on Benzidine Compounds

chromogen:	BZ	oTD	oDAD	DAF	TMB
wavelength (nm):	450	630	470	602	652
Chelator			ΔA/5 min.		
None ($FeCl_3$)	0.94	4.5	12	33	21
DHG	1.5	13	5.2	86	47
EDDA	0.36	3.4	8.5	69	34
NTA	0.091	0.67	2.8	31	5.1
NTP	0.071	0.25	0.86	6.4	1.5
EGTA	0.067	0.44	0.78	7.6	4.5
CIT	0.000	0.000	0.010	0.12	0.045
OX	0.000	0.000	0.014	0.099	0.035
DTPA	0.001	0.000	0.024	0.085	0.055
TTHA	0.000	0.000	0.024	0.047	0.027
EDTA	0.000	0.001	0.027	0.086	0.022
EDADP-ß	0.002	0.000	0.020	0.097	0.022
CDTA	0.000	0.000	0.016	0.033	0.009
EDADP-α	0.000	0.000	0.005	0.009	0.000
PP_i	0.000	0.000	0.000	0.000	0.000
EDDHA	0.000	0.000	0.002	0.000	0.000
DFA	0.000	0.000	0.000	0.000	0.000

Example 2

Effect of Chelators on Storage Stability of TMB/H_2O_2 Indicator Solution: 5-Day Incubations

o-Anisic acid (Aldrich Chemical Co.) was recrystallized from aqueous EDTA solution by the following procedure. 33.7 g (0.22 moles) of o-anisic acid were dissolved completely in 500 ml deionized H_2O brought to 88C on a magnetic stirring hot plate and immediately vacuum-filtered at room temperature through an 0.2 µm pore-size nylon filter, 47 mm in diameter (Rainin Instrument Co.). The filtrate was augmented with enough Na_2EDTA to give a final EDTA concentration of 10^{-3} M and re-heated to 88C to redissolve all of the o-anisic acid. This solution was covered and allowed to cool to room temperature over four hours without stirring, and then to 5C overnight. The resulting colorless crystals were vacuum filtered as above and washed with about 150 ml 5C deionized H_2O. They were dried for 5-6 hours at 50C in a vacuum oven at -22"Hg to yield 30.3 g (90%).

A chelator-free peroxidatic activity indicator solution was made by adjusting a solution of 0.30 g recrystallized o-anisic acid in 200 ml deionized H_2O to pH 4.00 with NaOH, adding 320 µl 0.2 M 3,3',5,5'-tetramethylbenzidine (TMB) (stock solution in N-methylpyrrolidone) slowly with stirring, and adding 68 µl of 30% (8.8 M) H_2O_2. This solution was immediately distributed among 50 ml screw-capped polypropylene centrifuge tubes containing 0.20 ml of 0.1 M stock chelator or 0.40 ml of 0.05 M stock chelator to create 20.0 ml volumes which were 10^{-2} M in o-anisate, 10^{-3} M in chelator, 0.32 mM in TMB, and 3.0 mM in H_2O_2, pH 4.0. The chelators used were DFA, EDDHA, EDADP-α, CDTA, PP_i, EDTA, and EDADP-ß; their stock solutions are described in Example 1, as are the sources of the chelators. As the purpose of this experiment was to compare the abilities of these chelators (those found to be most protective of TMB in Example 1) to block Fe(III)-catalyzed H_2O_2 oxidation of TMB on a time scale more nearly relevant to indicator solution storage stability, these solutions were augmented with 20 µl of freshly diluted 10^{-2} M $FeCl_3$ in H_2O to give a final Fe(III) concentration of 10^{-5} M, one tenth of that used in Example 1. The tubes were covered with aluminum foil and stored at room temperature (24-26C) in a dark enclosure except for the brief periods when they were assayed

spectrophotometrically for the appearance of the blue product of TMB oxidation (λ_{max} at 370 and 652 nm; low shoulder at 450 nm contributed by a small amount of the yellow product of TMB oxidation) and for sensitivity to horseradish peroxidase (HRP). The former assay was done in a 1 cm path-length cuvette balanced with H_2O. For the latter, 1.1 mg/ml HRP was diluted 1/100 and then another 1/501 into 0.10 M 2-(N-morpholino)ethanesulfonic acid, pH 6.0, in glass test tubes. Of the resulting 22 ng/ml stock HRP, 10 µl was diluted into 500 µl of indicator solution. A_{652} was then recorded for five minutes, and the catalytic rate was calculated as ΔA_{652}/5 min.

Figure 1 shows the kinetic traces for TMB oxidation over 5 days' storage of the indicator solution containing the seven different chelators. It suggests the following conclusions.

(1) Most of these traces show the surprising feature of a decay in the oxidation rate over a period of hours to days, depending on the chelator. Their concave-downward curvature is not predicted for a simple iron-catalyzed reaction, which should have an essentially constant rate (ascending linear kinetic trace) until much more of the TMB was oxidized. In these solutions, complete oxidation of TMB to the blue product should yield an A_{652} of 6.2; the equilibrium constant is very large, so that the reaction should go to completion unless something inhibits it.

(2) The simplest explanation for the level-off of the kinetic traces is that the oxidation product inhibits the oxidative catalyst (an Fe(III) chelate complex), presumably by binding to it. This inhibition process is at least as important as the initial oxidation rate, described in Example 1, for determining the ability of a chelator to protect a benzidine compound from oxidation. Fe(III)-chelate complexes differ in (a) intrinsic catalytic activity (initial rate), (b) the tightness of the inhibitory complex with oxidation product, which determines how high A_{652} rises before inhibition is complete, and (c) the apparent rate constant for formation of the inhibitory complex, which determines the time scale on which the trace levels off.

(3) Of the seven chelators tested here, DFA and EDDHA show comparable protective performance and are about five times more effective than CDTA and EDADP-α, which show comparable performance and are about three times more effective than EDTA and EDADP-ß, which show comparable

protection. These groupings and rankings are close to those shown by these chelators for initial oxidation rate in Example 1, except that EDADP-α groups here with CDTA rather than with DFA and EDDHA.

(4) Within these groups, CDTA shows a much higher initial rate than EDADP-α; and EDADP-ß shows a much higher initial rate than EDTA. However, once product inhibition has set in, the initially less protective chelator actually become more protective, because of a slow decline in A_{652} which is not simply explained by the product inhibition. This last phenomenon causes EDADP-ß to demonstrate improved performance over EDTA after several days of incubation.

(5) PP_i shows behavior with 10^{-5} M Fe(III) in o-anisate buffer inconsistent with its highly protective role with 10^{-4} M Fe(III) in piperazine buffer (Example 1). Instead of performing comparably to DFA and EDDHA, it is less protective than EDTA, having both an unexpectedly high initial rate and very slowly developing product inhibition. This anomaly is completely buffer dependent. Experiments like these but performed in pH 4.0 piperazine and citrate buffers show in the first case behavior comparable to CDTA and EDADP-α (but still worse than DFA and EDDHA, because of the slowness with which product inhibition develops), and in the second case behavior worse than TTHA and DTPA, which themselves are much less protective than EDTA (data not shown). Contrary to the predictions of Example 1, PP_i, is not generally an improvement in protection over EDTA as representative of the previously disclosed state of the art. However, it may still be an improvement in some buffers, such as piperazine and N-methylpiperazine. It has another potential advantage as well: relative resistance to damage by free radicals generated during transition-metal-ion catalyzed oxidations. The abundance of C-H bonds in the successful organic chelators renders them vulnerable to free-radical attack, so that their protective value might decline during prolonged storage.

With the exception of the pyrophosphate-chelated solution, these indicators reached their maximum accumulation of the blue product of TMB oxidation within four days of adding 10^{-5} M Fe(III). How did they perform with respect to the other indicator of storage stability, sensitivity to added HRP? After six days of incubation, none had lost more than 20% of the catalytic rate measured for a standard addition of HRP (to a final concentration in the cuvette of 0.43 ng/ml). These results confirm that the decay of the

TMB background oxidation rate during storage is not caused by substrate depletion or reaction equilibration.

As described in Example 3, the concentration of deliberately introduced Fe(III) here is two orders of magnitude higher than that expected in indicator solutions prepared with an eye toward excluding iron. It is reasonable to expect the latter solutions to have storage lifetimes two orders of magnitude longer than the present solutions would show, yielding in addition much lower A_{652} values than displayed in Figure 1. The self-quenching nature of the background reaction is a special property of benzidine compounds, not seen with chromogenic electron donors generally [see Example 6].

Example 3

Transition-Metal-Ion Contamination of Buffer Components and Chelators

Samples of commercially supplied reagent-grade organic and inorganic compounds commonly used in making peroxidatic activity indicator solutions were subjected to analysis for transition metal trace contaminants by ion-coupled plasma (ICP) atomic-emission spectroscopy. The pure solids and liquids were prepared for ICP analysis by either dissolution in 4% HCl or wet-ash digestion in H_2SO_4/HNO_3, using methods standard in the art of instrumental trace-element analysis. Some samples of o-anisic acid and 1,2,4,5-benzene tetracarboxylic acid were first recrystallized as described in Example 2, sometimes excluding EDTA. Table II summarizes some of the analytical results as supplied in units of parts per million. Here "detection limit" is the smallest quantity distinguishable from background, approximately twice the background signal; and "limit of detectability" is five times the detection limit, or about ten times the background signal. Values above the limit of detectability have relative uncertainties below about 20%, whereas values between the limit of detectability and the detection limit may have relative uncertainties as high as 100%.

Table II suggests several conclusions.

(1) Iron is the principal oxidatively active transition metal which exists as a trace contaminant in reagent-grade chemicals. Occasional samples

contain significant levels of chromium, but the other metals generally are found near or below the detection limit of ICP and well below the level of iron contamination. For this reason, it is practical to focus on iron when studying the catalytic properties of transition metal ion chelates as in Examples 1 and 2.

(2) Recrystallization of aromatic carboxylic acids such as o-anisic acid and 1,2,4,5-benzene tetracarboxylic acid in the absence of a strong chelator reduces transition metal ion contamination modestly or marginally; inclusion of EDTA in the recrystallization solvent dramatically improves performance.

(3) Commercial reagent-grade citric acid is remarkably transition-metal-ion free despite the chelating properties of citrate. Strong chelators, such as EDTA, DTPA, and TTHA, are not more contaminated than most of the other reagents analyzed; and DTPA is remarkably pure.

The greatest practical value of the data in Table II derives from estimation of the concentrations of contaminating iron in peroxidatic activity indicator solutions of specified composition, assuming that contributions from deionized H_2O, TMB, and H_2O_2 are negligible. Table III shows such estimates for several indicator solutions for which storage stability data are available. Use of citrate instead of succinate as buffer acid and use of 10^{-3} M EDTA instead of 10^{-2} M EDTA as chelator suffice to keep the Fe concentration below 10^{-7} M.

Figures 2 and 3 show partial results from long-term storage-stability studies varying either the EDTA concentration (in 10^{-2} M citrate buffer) or the buffer acid (citrate or succinate) in 10^{-3} M EDTA. For these experiments, the indicator solutions (10^{-2} M citrate or succinate, 10^{-3} or 10^{-2} M EDTA, 0.3 mM TMB, 3.0 mM H_2O_2, adjusted to pH 3.9-4.0 with solid $Na_2CO_3 \cdot H_2O$) were made and stored in polystyrene containers which had been incubated at least two days in 10^{-2} M Na_2 EDTA adjusted to pH 7.2 and had been rinsed with deionized H_2O before use. Incubations were done in the dark in 25C or 37C thermostated incubators. Assays for TMB oxidation product and for sensitivity to HRP catalysis were performed as in Example 2, except that enzyme activity was recorded as initial rate rather than the total A_{652} increase over five minutes. Figure 2 shows that the succinate buffer, estimated in Table 3 to contain 0.12 μM Fe, developed almost three times the A_{652} value of the citrate

buffer, estimated to contain 0.03 μM Fe. Figure 3 shows that within three months of formulation, an indicator solution containing 10^{-2} M EDTA and estimated to contain 0.13 μM Fe had lost a significant fraction of its original ability to detect HRP, whereas an otherwise identical solution containing 10^{-3} M EDTA and approximately 0.03 μM Fe still possessed full activity at four months. These storage stability studies were stopped at four months for lack of material, not because of failure of all formulations. The performance of 10^{-2} M citrate, 10^{-3} M EDTA shown here has been surpassed by a similar indicator solution containing 10^{-2} M citrate, 10^{-3} M CDTA, which had an A_{652} of .014 and retained 85% of initial catalytic activity after seven months storage in the dark at room temperature.

These experiments suggest that stability of pH 4 TMB-H_2O_2 peroxidatic activity indicator solutions depends on reducing iron contamination levels below approximately 10^{-7} M, the exact threshold probably depending on choice of chelator. Furthermore, such levels can be reached without strenuous application of metal-removal procedures. Added measures, such as preparation of metal-free buffer acid or chelator, and treatment of indicator solutions with an immobilized chelator, should improve the margin of safety. There are several reasons why the peroxidatic activity indicator solutions of the invention which employ a benzidine-compound chromogen are expected to show practical storage lifetimes much longer than the maximum of seven months reported here.

(a) The successful TMB-containing solutions described in this example were losing activity and gaining absorbance so slowly that the ultimate storage lifetime would have been longer than the reported four and seven months.

(b) Of the benzidine compounds tested in Table I, only DAF was consistently more sensitive to Fe(III) than TMB, and only by a small margin. Therefore, measures which serve to meet the storage-lifetime conditions of the invention for TMB should exceed the storage-lifetime conditions for most of the other benzidine compounds.

(c) The successful TMB-containing solutions described in this example were formulated at the pH value and substrate concentrations giving greatest sensitivity to HRP, not greatest storage stability. As shown in Figure 4, pH 4 indicator solutions develop more background color than indicator

solutions at higher pH values, such as 5. Therefore, storage-lifetime should be extended simply by raising the indicator solution pH from the value of 4 used here. The TMB concentration used in this example is near the solubility limit. Lower concentrations, while giving lower sensitivity to HRP, also would have longer storage lifetimes. Likewise, lower H_2O concentrations than the 3 mM value used in this example would result in longer storage-lifetimes at some cost with respect to assay sensitivity.

(d) The chelators used in this example, EDTA and CDTA, while effective in reducing the background reaction, are not as effective as the best ones reported in Table I, EDDHA and DFA. Therefore, simply replacing them with EDDHA or DFA should substantially extend storage-lifetime.

TABLE II

Transition-Metal Levels in
Indicator Solution Components

(all values as ppm, or μg metal/g sample; < indicates no signal above the detection limit; brackets indicate a signal between the detection limit and the limit of detectibility)

Metal:	Co	Cr	Cu	Fe	Mn	Ni
Detection Limit:	0.2	0.2	0.1	0.1	0.04	0.4
Reagent						
Succinic acid	<	<	<	5.5	<	<
Citric acid·H_2O	<	<	<	[0.4]	<	<
Fumaric acid	<	[0.6]	<	1.6	<	<
Benzoic acid	<	[0.3]	<	4.7	[0.08]	<
o-Anisic acid	<	<	<	1.5	<	<
Recryst. o-anisic acid*	<	[0.3]	<	0.7	<	<
1,2,4,5-Benzene tetracarboxylic acid	[0.4]	2.1	[0.4]	1.1	[0.08]	[0.8]
Recryst. 1,2,4,5-benzene tetra-carboxylic acid*	[0.4]	1.6	[0.3]	1.3	[0.1]	<
Recryst. (from EDTA) 1,2,4,5-benzene tetracarboxylic acid	[0.5]	[0.7]	[0.3]	[0.2]	[0.08]	[0.8]
$Na_2CO_3·H_2O$	<	<	<	[0.3]	<	<
EDTA (free acid)	<	<	<	1.6	<	<
Na_2 EDTA·$2H_2O$	[0.4]	[0.4]	[0.2]	1.5	[0.7]	<
Na_4 EDTA·$2.5H_2O$	[0.5]	[0.8]	[0.3]	1.4	[0.18]	[0.5]
DTPA	<	<	<	[0.2]	<	<
TTHA	<	7.1	<	2.0	<	[0.7]

*These recrystallizations lacked EDTA

TABLE III

Fe Concentrations in Some Substrate Formulations
Already Tested for Storage Stability

(ignoring possible contributions from Na_2CO_3, TMB, H_2O_2, or H_2O, expected to be minor)

	Buffer Composition	µM Fe	
(a)	10^{-2} M succinate	0.12	} 0.13
	10^{-3} M Na_2 EDTA	0.011	
(b)	10^{-2} M succinate	0.12	} 0.23
	10^{-2} M Na_2 EDTA	0.11	
(c)	10^{-2} M citrate	0.02	} 0.03
	10^{-3} M Na_2 EDTA	0.011	
(d)	10^{-2} M citrate	0.02	} 0.13
	10^{-2} M Na_2 EDTA	0.11	

Example 4

Effects of pH and Temperature on Storage Stability
of TMB-H_2O_2 Peroxidatic Activity Indicator Solutions

To study pH dependence, citric acid and Na citrate stock solutions, stored in glass, were mixed to get pH values of 3.59, 3.96, 4.38, 4.70, and 4.97 at room temperature with a total citrate concentration of 10^{-2} M. Solid Na_2 EDTA·$2H_2O$ and stock solutions of TMB and H_2O_2 were added to achieve 10^{-3} M EDTA, 0.10 mM TMB and 3.0 mM H_2O_2 at final pH values, given in Figure 4, which were marginally more alkaline than shown above. These indicator solutions were incubated in a dark incubator thermostated at 25C in screw-capped polyvinyl chloride test tubes, measuring A_{652} at intervals over several days. To study temperature dependence, 10^{-2} M succinate, 10^{-3} M EDTA, 0.3 mM TMB, 3.0 mM H_2O_2, pH 4.1 was prepared and stored in polystyrene containers previously soaked in 10^{-2} M NaEDTA, pH 7.2. The tubes were stored

in the dark in thermostated incubators at 4C, 25C, 37C, and 46C, withdrawing small aliquots for measuring A_{652} and A_{285} at regular intervals. pH was measured at 1C, 27C, and 41C, giving pH values of 3.90, 4.11, and 4.25, respectively. Therefore pH rises by 0.01 pH unit for every 1C increase in temperature. A single measurement of sensitivity to HRP, done after 256 hours of incubation, showed no loss of activity which could not be attributed to modest (<20%) loss of TMB (calculated from A_{285}), which varied randomly among tubes and probably was caused by adsorption to the walls of the tubes. Figure 4 shows the striking pH dependence of TMB oxidation over a period of three days at 25C. Lower pH values strongly promote TMB oxidation, apparently not by changing the rate constant for approach to the product-inhibited state described in Example 2, but rather by increasing the total amount of the blue oxidation product formed before the decay in oxidative activity is complete. The simplest explanation is that TMB becomes increasingly protonated as pH is dropped below 5, and that protonated TMB competes with the cationic oxidation product for binding to the anionic Fe·EDTA chelate complex which is the probable oxidative catalyst. Such a competition between reactant and product for catalyst would weaken the product inhibition, allowing more product to be formed. From a practical viewpoint, Figure 4 suggests that indicator solution storage stability is more easily obtained at pH 5 than at lower pH values, although at some cost in indicator sensitivity because TMB is four times more soluble at pH 4 and because HRP is significantly more active at pH 4 than at pH 5. Alternatively, measures which achieve practical storage stability at pH 4 should provide a greater margin of security at higher pH values.

The temperature dependence of the background reaction, shown in Figure 5, allows the remarkable conclusion that higher storage temperatures actually stabilize TMB and H_2O_2 toward oxidation catalyzed by trace levels of iron, in this case chelated by EDTA. Higher temperatures also accelerate the approach of the background reaction to the product-inhibited state as is theoretically expected. The anomalous effect of temperature on plateau value of A_{652} probably arises from the effect of temperatures on pH combined with the effect of pH on TMB oxidation shown in Figure 4. These indicator solutions had a pH of 4.1 at 25C, and should have pH values of 3.9 at 4C and 4.3 at 46C. The practical significance of these results is that cold storage,

commonly used to prolong the lifetime of enzyme substrate solutions, is counterproductive for TMB-H_2O_2 indicator solutions. Not only is room temperature storage adequate, but there is no value to protecting such indicator solutions from elevated temperatures during shipping and storage. Such solutions may be especially suited to diagnostic applications in tropical countries. Figure 5 also shows the effect of a down-shift in temperature from 46C to 25C; the indicator solution immediately began to re-equilibrate to the degree of catalyst product inhibition expected at 25C.

Example 5

Effect of Container Composition on Storage Stability of TMB-H_2O_2 Peroxidatic Activity Indicator Solutions

Three indicator solutions were prepared which contained 10^{-2} M Na citrate, 0.3 mM TMB, 3.0 mM H_2O_2, pH 4.0. One contained citrate buffer which had stood three days at room temperature in a glass container before adding substrates; this indicator was stored in glass test tubes during the 3 day incubation at room temperature. The other two contained citrate buffer which had stood one day at room temperature in a polyvinylchloride container; and after adding substrates, one was incubated in glass and one was incubated in polypropylene. Aliquots were withdrawn at intervals during incubation (in the dark) for measurement of A_{652}. Figure 6 shows kinetic traces for TMB oxidation during storage. The trace indicated by A shows storage of buffer in glass and indicator incubation in glass. The trace indicated by B shows storage of buffer in polyvinylchloride and indicator incubation in glass. The trace indicated by C shows storage of buffer in polyvinylchloride and indicator incubation in polypropylene. The figure suggests that prior buffer storage in glass causes a dramatic increase in transition metal ion content, and that incubation of the complete indicator solution in glass releases more catalytically active metal. Preparation and storage in plastic minimize the extent of background oxidation of TMB by H_2O_2. Such container effects are likely to vary widely depending on the history of the container, not controlled in this experiment. The glass containers were reused from a laboratory supply after being cleaned with hot detergent, with tap water and deionized water rinses.

The plastic containers were previously unused disposable items. Chelator (other than citrate buffer) was omitted from this experiment because it tends to suppress the effects of other variables. However, differences parallel to those in Figure 6 were found when 10^{-3} M EDTA was included in the indicator.

Example 6

Improvement of Storage Stability of oPD-H_2O_2 Indicator Solutions for Peroxidatic Activity

The o-phenylenediamine (oPD) was from three sources: commercially supplied oPD$(HCl)_2$ (Sigma Chemical Co.), commercially supplied zone-refined oPD (Aldrich Chemical Co., Gold Label), and oPD sublimed by the inventor from commercially supplied less pure chromogen (Aldrich Chemical Co.). The latter two purified preparations were colorless and functionally equivalent. The cruder commercial oPD$(HCl)_2$ and oPD were tan or violet in color. Sublimation was performed at 89-100C. Re-sublimation of once-purified oPD did not result in improved storage stability of indicator solutions.

In an experiment to compare the effectiveness of various chelators on storage stability of oPD-H_2O_2, indicator solutions were prepared containing 10^{-2} M Na citrate, 15 mM oPD$(HCl)_2$, 5.5 mM H_2O_2, pH 5.0, plus 10^{-3} M DFA, DTPA, DHG, NTP, EGTA, or EDTA. A control preparation contained no chelator except the citrate buffer, and another solution contained 10^{-4} M DFA. The indicator solutions were incubated at room temperature in foil-covered polypropylene tubes which had been soaked for at least two days in 10^{-2} M EDTA, pH 7.5, and rinsed with deionized H_2O. Aliquots were withdrawn at measured intervals after addition of the H_2O_2 to measure A_{450}, the absorbance λ_{max} for oxidized oPD.

Table IV reports on the relative effectiveness of these chelators in blocking the background oxidation of oPD by H_2O_2, catalyzed by uncontrolled and unmeasured trace transition metal contaminants in the solutions. It shows that DTPA marginally improved and DFA significantly improved on the protective function of citrate alone. The other chelators were worse, or in the case of EDTA, much worse, than citrate alone. Prior experiments screening chelators in the presence of a known concentration (0.05 mM) of Fe(III),

analogous to the experiments in Example 1, had shown that chelators such as CDTA, EDADP-α, EDADP-ß, and EDDHA, which very effectively protected benzidine compounds in Example 1, had less protective value with oPD than any of the chelators in Table IV except EDTA, which was the worst of all of the chelators tested. Kinetic traces of the citrate-alone and citrate + EDTA reactions of Table IV are shown in Figure 7. They show that unlike TMB, oPD shows little sign of any sort of product inhibition which might serve to reduce the deleterious effects of transition-metal contaminants.

Also shown in Figure 7 are kinetic traces from several background oxidation reactions in indicator solutions like those described above except that zone-refined oPD was used. The data for commercial and repurified oPD stored under various conditions are compared in traces D-H. D indicates commercial oPD with 10^{-2} M citrate + 10^{-3} M EDTA; E indicates commercial oPD with 10^{-2} M citrate; F indicates repurified commercial oPD with 10^{-2} M citrate; G indicates repurified commercial oPD with 10^{-2} M citrate + 10^{-4} M DFA; and H indicates repurified commercial oPD with 10^{-2} M citrate + 10^{-3} M DFA. These traces confirm that DFA protects oPD in a concentration-dependent manner, and also show that oPD repurification is as effective in reducing the background reaction as adding DFA. Additionally, the traces for repurified oPD show a lag phase before the onset of oPD oxidation, a phenomenon which serves to enhance storage lifetime.

The DFA effect is the major evidence that most of the indicator lability may be attributed to transition metal ion catalysis. There is no reason to suspect that there is a measurable uncatalyzed component of the background reaction which limits the potential gains from further reduction of the level of transition metal contamination.

Figures 8A and 8B show a final set of experiments employing approximately optimized indicator solutions to test the effect of pH on storage stability. The optimized conditions used sublimed oPD at a concentration of 3.3 mM, H_2O_2 at a concentration of 5.0 mM, piperazine as the buffer base (rather than NaOH), and citrate or DTPA as the buffer acid. 10^{-2} M sublimed piperazine was adjusted to the desired pH by adding solid DTPA or citric acid monohydrate. Piperazine had been sublimed at 80-90C. All incubations were in foil-wrapped polypropylene tubes stored in the dark. Figures 8A and 8B differ

by the storage temperature of 25C and 4C, respectively. The data from various storage conditions are compared in traces I-L. In each Fig 8A and 8B, I represents piperazine + DTPA, pH 5.0; J represents piperazine + citrate, pH 5.0; K represents piperazine + citrate, pH 4.4; L represents piperazine + citrate, pH 4.0. The kinetic traces in Figure 8A show that A_{450} can be maintained below 0.010 for 12-14 hours, or well over one work day, at room temperature if the buffer contains citrate. pH 4.0-4.4 is somewhat preferable to pH 5.0. DTPA is slightly less protective than citrate at pH 5.0. Figure 8B shows that storage at 5C extends the storage lifetime, as the A_{450} of the solution remains less than 0.010 for 7.5-9.6 days, or well over one work week, at pH 4.4-4.0. Even at pH 5, the A_{450} can be kept below 0.010 for almost six days in citrate. Several features appear to be responsible for the storage stability performance depicted in Figure 8, which is an improvement over the accepted art.

(a) The oPD used had been repurified by sublimation and was colorless.

(b) The buffer was made from components known or expected to be relatively free of contaminating transition metal ions: sublimed piperazine, citrate (see Example 3), and DTPA (see Example 3).

(c) The pH range was lower than than commonly used with oPD as a chromogen, pH 5-6.

(d) The oPD concentration was reduced to a value near its Km with HRP. The background reaction rate is expected to be approximately proportional to oPD concentration, whereas the HRP-catalyzed rate shows the normal Michaelian oPD concentration dependence. Lowering [oPD] from 15 mM to 3.3 mM should lower the background reaction rate more than 75% while lowering the HRP-catalyzed rate (and therefore the sensitivity to HRP) less than 50%.

The optimal reaction conditions do not represent a single set of concentrations, pH, and buffer salt composition, but rather a range of values for these variables. HRP activity is maximum around pH 4.5, but the loss of sensitivity to HRP at pH 4.0 relative to pH 4.4 is only about 25%; at pH 5.0 sensitivity to HRP has declined only 8% from the value at pH 4.4. oPD and H_2O_2 concentrations should be in the vicinity of the respective Km values for the enzyme being assayed (usually HRP), but exact values are not critical.

However, because oPD appears to lack the product-inhibition phenomenon which contributes greatly to the storage stability of indicator solutions using benzidine compounds as chromogens, most progress in further stabilization of oPD-H_2O_2 indicator solutions must depend on application of strenuous efforts to reduce transition metal ion contamination, such as treatment of indicator solutions with immobilized chelators or synthesis or repurification of buffer acid under metal-free conditions.

TABLE IV

Effect of Chelators on oPD + H_2O_2 Background Reaction in 10^{-2} M Na Citrate, pH 5.0

Chelator	A_{450} After 24 Hr Incubation*
None	.290
10^{-4} M DFA	.088
10^{-3} M DFA	.075
10^{-3} M DTPA	.263
10^{-3} M DHG	.307
10^{-3} M NTP	.302
10^{-3} M EGTA	.321
10^{-3} M EDTA	1.566

*A_{450} at start of reaction = .010

The invention has been described in detail with particular reference to preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A peroxidatic activity indicator solution composition comprising

(a) a buffer;

(b) a chelator selected from the group consisting of ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDHA), ethylenediaminetetraacetic acid (EDTA), trans-1,2-diaminocyclohexane-N,N,N'N'-tetraacetic acid (CDTA), desferrioxamine B (DFA), ethylenediamine-N,N'-diacetic acid-N,N'-di-α-propionic acid (EDADP-α), ethylenediamine-N,N'-diacetic acid-N,N'-di-ß-propionic acid (EDADP-ß), ethylenediamine-tetra-ß-propionic acid (EDTP-ß), ethylenediamine-tetra-α-propionic acid (EDTP-α), N,N'-bis(2-hydroxybenzyl)ethylenediamine diacetic acid (HBED), and pyrophosphate (PP_i);

(c) a hydroperoxide; and

(d) a benzidine compound selected from the group consisting of benzidine, o-tolidine, o-dianisidine, 3,3',5,5'-tetramethylbenzidine (TMB), 3,3'-diethylbenzidine, and 2,7-diaminofluorene,

said composition having a storage lifetime of at least about six months at a temperature of between about 0C and about 40C, and a total concentration of oxidatively active transition metals less than about 10^{-7} M.

2. The composition of claim 1, wherein said hydroperoxide is selected from the group consisting of hydrogen peroxide and urea hydrogen peroxide.

3. The composition of claim 1, wherein said chromogenic electron donor is TMB.

4. The composition of claim 1, wherein said buffer maintains a pH of between about 4 and about 8.

5. The composition of claim 1, wherein said buffer is selected from the group consisting of citrate, succinate, benzoate and o-anisate.

6. A peroxidatic activity indicator solution composition comprising a buffer, a hydroperoxide, and o-phenylenediamine (oPD), said composition having a storage lifetime of at least about 36 hours at 25C or 2 weeks at 5C, and a total concentration of oxidatively active transition metal less than about 1×10^{-7} M.

7. The composition of claim 6, wherein said oPD is selected from the group consisting of sublimed oPD and zone refined oPD.

8. The composition of claim 6, wherein said buffer has a pH betweem about 10 and about 4.

9. The composition of claim 6, wherein the concentration of said oPD is below about 5 mM.

10. The composition of claim 6, wherein said hydroperoxide is selected from the group consisting of hydrogen peroxide and urea hydrogen peroxide.

11. The composition of claim 6 that further comprises a chelating agent selected from the group consisting of DTPA and DFA.

12. The composition of claim 10, wherein said hydroperoxide is at a concentration of less than about 5 mM.

13. The composition of claim 6, wherein said buffer comprises a compound selected from the group consisting of citrate, piperazine and N-methylpiperazine.

14. A composition comprising a chromogenic electron donor selected from the group consisting of benzidine, o-tolidine, o-dianisidine, 3,3',5,5'-

tetramethylbenzidine (TMB), 3,3'-diethylbenzidine, and 2,7-diaminofluorene in combination with a chelating agent selected from the group consisting of DFA, EDDHA, HBED, EDTP-α, EDTP-ß, CDTA, EDADP-α, EDADP-ß and PP_i.

15. The composition of claim 14 that comprises a hydroperoxide.

16. The composition of claim 15, wherein said hydroperoxide is selected from the group consisting of hydrogen peroxide and urea hydrogen peroxide.

17. The composition of claim 14 that comprises a buffer of pH between about 4 and about 8.

18. The composition of claim 17, wherein said buffer is selected from the group consisting of citrate, succinate, benzoate, o-anisate, piperazine, N-methyl piperazine, fumarate and pyromellitate.

19. The composition of claim 14 further comprising an electron-donor substrate for an oxidase enzyme.

20. The composition of claim 14 further comprising an organic cosolvent.

21. The composition of claim 20, wherein said organic cosolvent is selected from the group consisting of N-methyl-2-pyrrolidone, ε-caprolactam, N-2-hydroxyethyl-2-pyrrolidone, N-ethyl-2-pyrrolidone, and N-cyclohexyl-2-pyrrolidone.

22. A process for preparing a peroxidatic activity indicator solution with increased storage lifetime, comprising the steps:

(a) treating water to render said water substantially metal-free;

(b) combining said water prepared in step (a) with a buffer, a

hydroperoxide, and a chromogenic electron donor under metal-free conditions;

(c) storing said solution prepared in step (b) under metal-free conditions; and

(d) shielding said solution prepared in step (c) from ultraviolet radiation.

23. The process of claim 22 that further comprises filtering said solution prepared in step (b) through a membrane having a nominal pore size less than about 2 μm.

24. The process of claim 23, wherein said nominal pore size is less than about 0.02 μm.

25. The process of claim 22, wherein said buffer or said chromogenic electron donor has been repurified by a method selected from the group consisting of distillation, sublimation, zone refinement, and recrystallization in the presence of a chelating agent.

26. The process of claim 22, wherein said chromogenic electron donor is a benzidine compound.

27. The process of claim 26 that further comprises adding at step (b) a chelating agent selected from the group consisting of DFA, EDDHA, HBED, EDTP-α, EDTP-ß, EDADP-α, EDADP-ß, CDTA, EDTA and PP_i.

28. The process of claim 26 that further comprises adding at step (b) a chelating agent selected from the group consisting of EDTA, CDTA, EDADP-ß EDADP-α, EDTP-α, and EDTP-ß, and wherein said chelating agent has been purified by a method selected from the group consisting of recrystallization as a sodium salt from H_2O/ethanol and precipitation as a free acid by acidification of an aqueous solution of the sodium or potassium salt.

29. The process of claim 28, wherein said purification is performed in the presence of an agent selected from the group consisting of DFA, HBED, and EDDHA.

30. The process of claim 26 that further comprises adding at step (b) a chelating agent selected from the group consisting of EDTA, CDTA, EDADP-α, EDADP-ß, EDTP-α, EDTP-ß, HBED, and EDDHA, wherein said chelating agent has been synthesized under metal-free conditions.

31. The process of claim 26, wherein said buffer is selected from the group consisting of citrate, succinate, benzoate and o-anisate.

32. The process of claim 22, wherein said hydroperoxide is selected from the group consisting of hydrogen peroxide and urea hydrogen peroxide.

33. The process of claim 22, wherein said chromogenic electron donor is oPD.

34. The process of claim 33 that further comprises adding in step (b) a chelating agent selected from the group consisting of DFA and DTPA.

35. The process of claim 34, wherein said DTPA has been purified by a method selected from the group consisting of recrystallization as a sodium salt from H_2O/ethanol and precipitation as a free acid by acidification of an aqueous solution of the sodium or potassium salt.

36. The process of claim 35, wherein said recrystallization and precipitation is performed in the presence of DFA, HBED, or EDDHA.

37. The process of claim 35, wherein said DTPA is synthesized under metal-free conditions.

38. The process of claim 22, wherein said solution is stored in a non-metal container selected from the group consisting of plastic and a non-

metal container which has been contacted with a solution effective for dissolving transition metals.

39. The process of claim 22, wherein said solution is stored in contact with a chelating solid.

40. The process of claim 22, wherein any liquid combination of at least two of the components of said water, said buffer, and said chromogenic electron donor is treated with an agent selected from the group consisting of an ion-exchange solid and a chelating solid.

41. The process of claim 40 wherein said ion exchange solid is selected from the group consisting of Duolite C264® Amberlite 200®, Amberlyst 15®, activated basic alumina, and activated silica.

42. The process of claim 40, wherein said chelating solid is selected from the group consisting of Duolite ES346®, Duolite ES466®, Duolite ES467®, Chelite C®, Chelite P®, Chelite N®, Amberlite IRC-718®, poly(acrylamidoxime) resin, Chelex-100®, and Thoron® in combination with an anion exchange solid.

43. A process of storing a peroxidatic activity indicator solution composition containing a buffer, a hydroperoxide and a chromogenic electron donor, comprising the steps:

(a) contacting said composition with a container which is substantially metal-free; and

(b) shielding said composition from ultraviolet radiation.

44. The process of claim 43, wherein said container is plastic.

45. The process of claim 43, wherein said container is treated with a solution that dissolves transition metals.

46. The process of claim 45, wherein said solution contains an agent selected from the group consisting of HNO_3, HCl, and H_2SO_4.

47. The process of claim 45, wherein said solution contains a chelating agent.

48. A kit for performing assays which utilize peroxidatic activity for detection, said kit comprises a peroxidatic activity indicator solution of claim 1.

49. The kit of claim 48, wherein said buffer is selected from the group consisting of citrate, succinate, benzoate and o-anisate.

50. The kit of claim 48, wherein said hydroperoxide is selected from the group consisting of hydrogen peroxide and urea hydrogen peroxide.

51. The kit of claim 48, wherein said assay is an analyte-specific binding assay.

52. The kit of claim 48, wherein said assay is for the detection of a peroxidatic catalyst selected from the group consisting of blood and hemoglobin.

53. The kit of claim 48, wherein said assay is used histochemically or cytochemically.

54. A kit for performing assays which utilize peroxidatic activity for detection comprising:

(a) an aqueous composition of a buffer and a hydroperoxide; and

(b) solid oPD;

wherein mixing of said aqueous composition and said solid oPD results in a peroxidatic activity indicator solution having a storage lifetime of at least

about 36 hours at 25C or 2 weeks at 5C, and a total concentration of oxidatively active transition metals less than about 1×10^{-7} M.

55. The kit of claim 54, wherein said buffer is citrate.

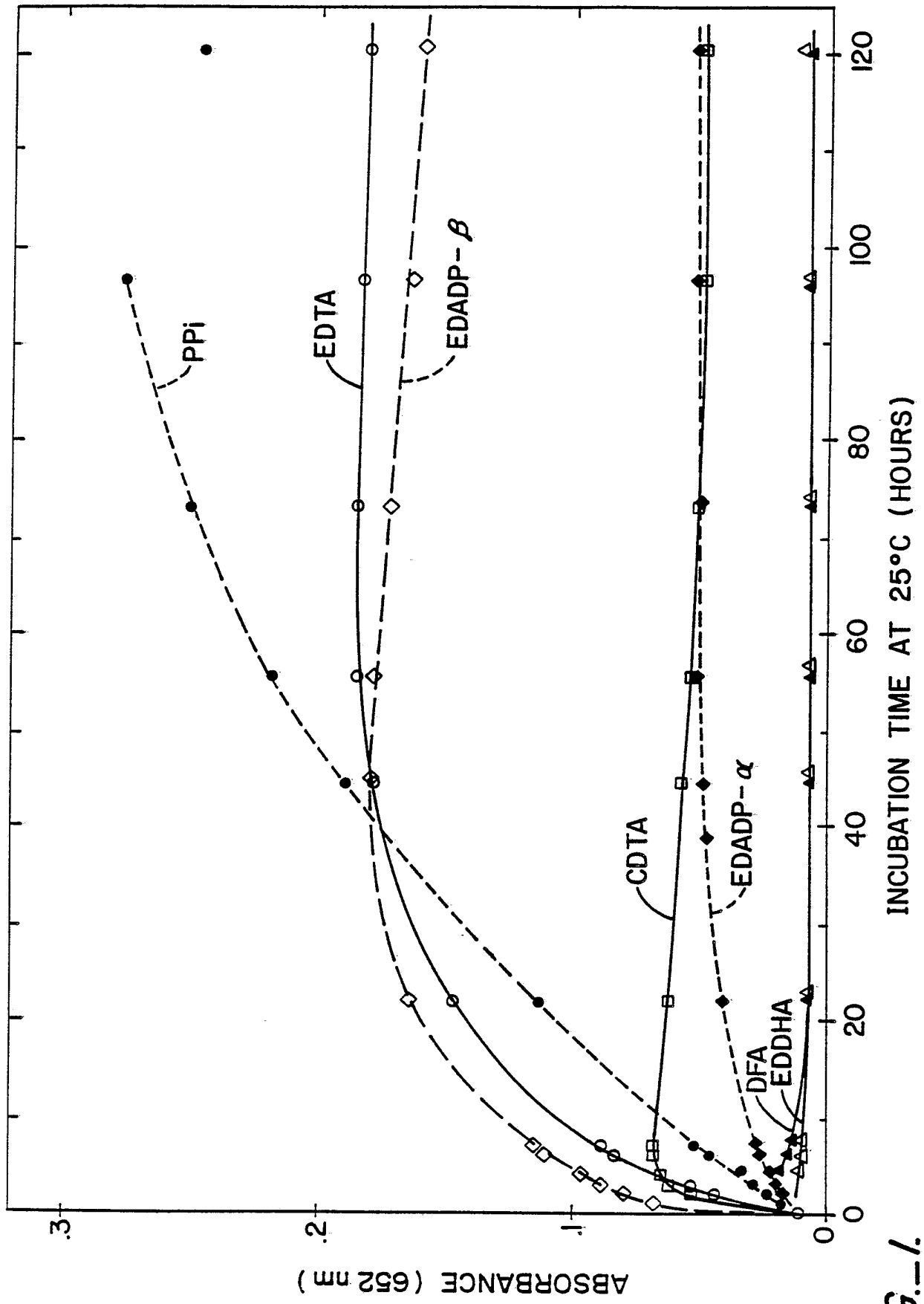
56. The kit of claim 54, wherein said hydroperoxide is selected from the group consisting of hydrogen peroxide and urea hydrogen peroxide.

57. The kit of claim 54, wherein said aqueous composition further comprises a chelating agent selected from the group consisting of DTPA and DFA.

58. The kit of claim 54, wherein said aqueous composition further comprises a buffer selected from the group consisting of piperazine and N-methylpiperazine.

59. The kit of claim 54, wherein said assay is an analyte-specific binding assay.

60. The kit of claim 54, wherein said assay is for the detection of a peroxidatic catalyst selected from the group consisting of blood and hemoglobin.


ABSORBANCE (652 nm)
0
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.2
.3
INCUBATION TIME AT 25°C (HOURS)
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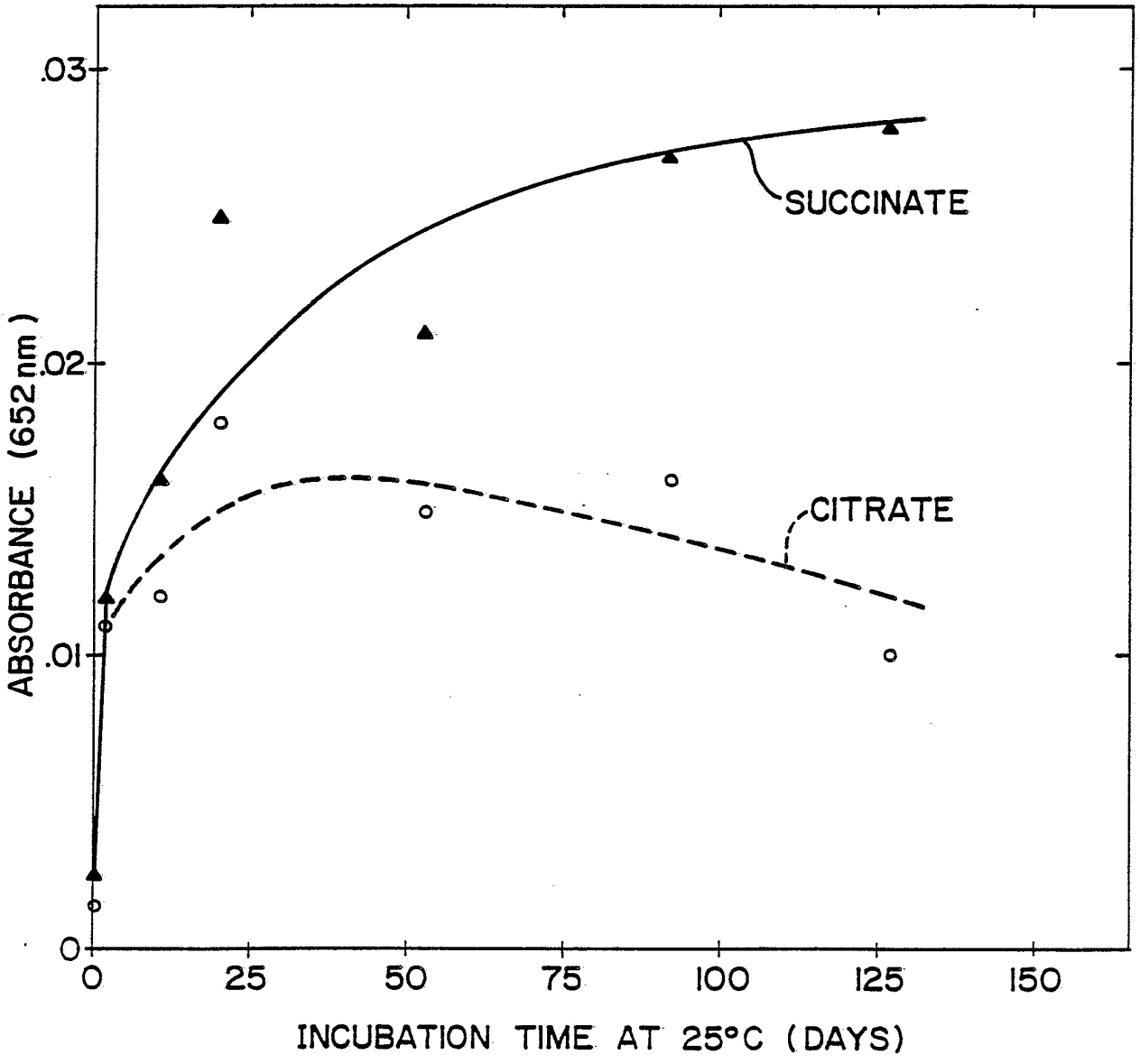
20
40
60
80
100
120
PPi
EDTA
EDADP-β
CDTA
EDADP-α
DFA
EDDHA

FIG._1.


.03
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.01
0
ABSORBANCE (652 nm)
SUCCINATE
CITRATE
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25
50
75
100
125
150
INCUBATION TIME AT 25°C (DAYS)

FIG._2.

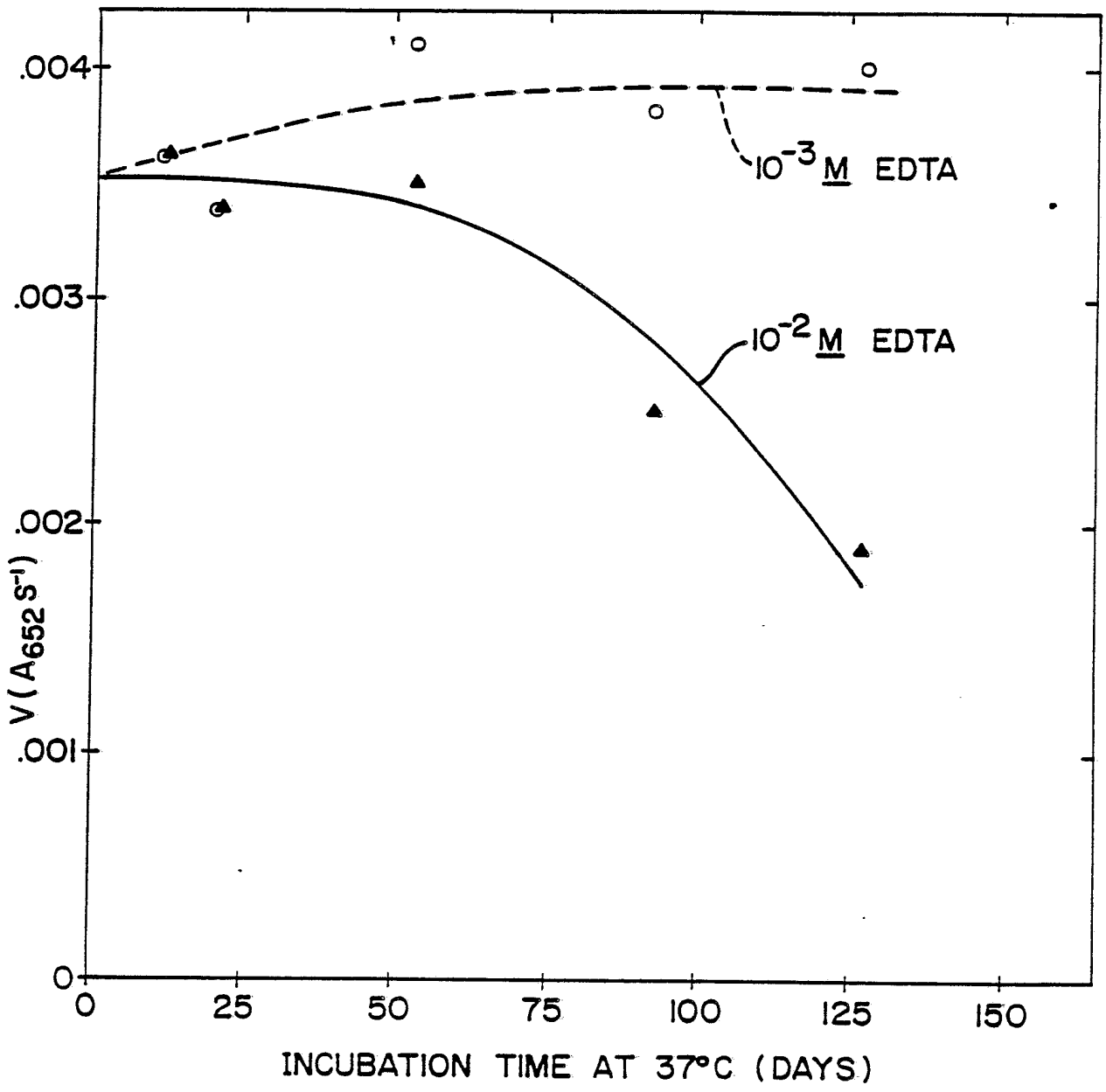
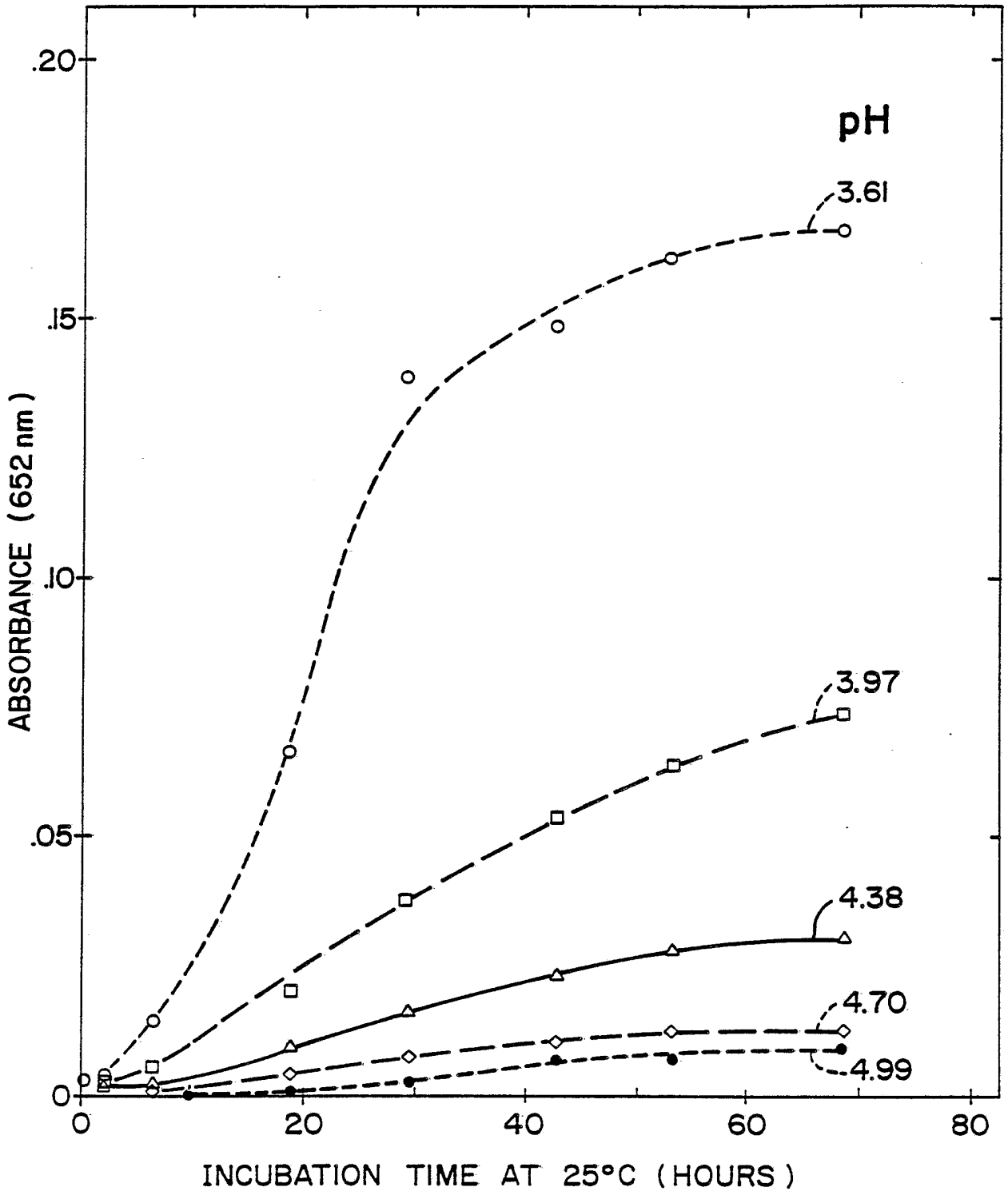

.004
.003
.002
.001
0
V(A652 S-1)
10-3 M EDTA
10-2 M EDTA
0
25
50
75
100
125
150
INCUBATION TIME AT 37°C (DAYS)

FIG._3.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.05
0
ABSORBANCE (652 nm)
pH
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3.97
4.38
4.70
4.99
0
20
40
60
80
INCUBATION TIME AT 25°C (HOURS)

FIG._4.

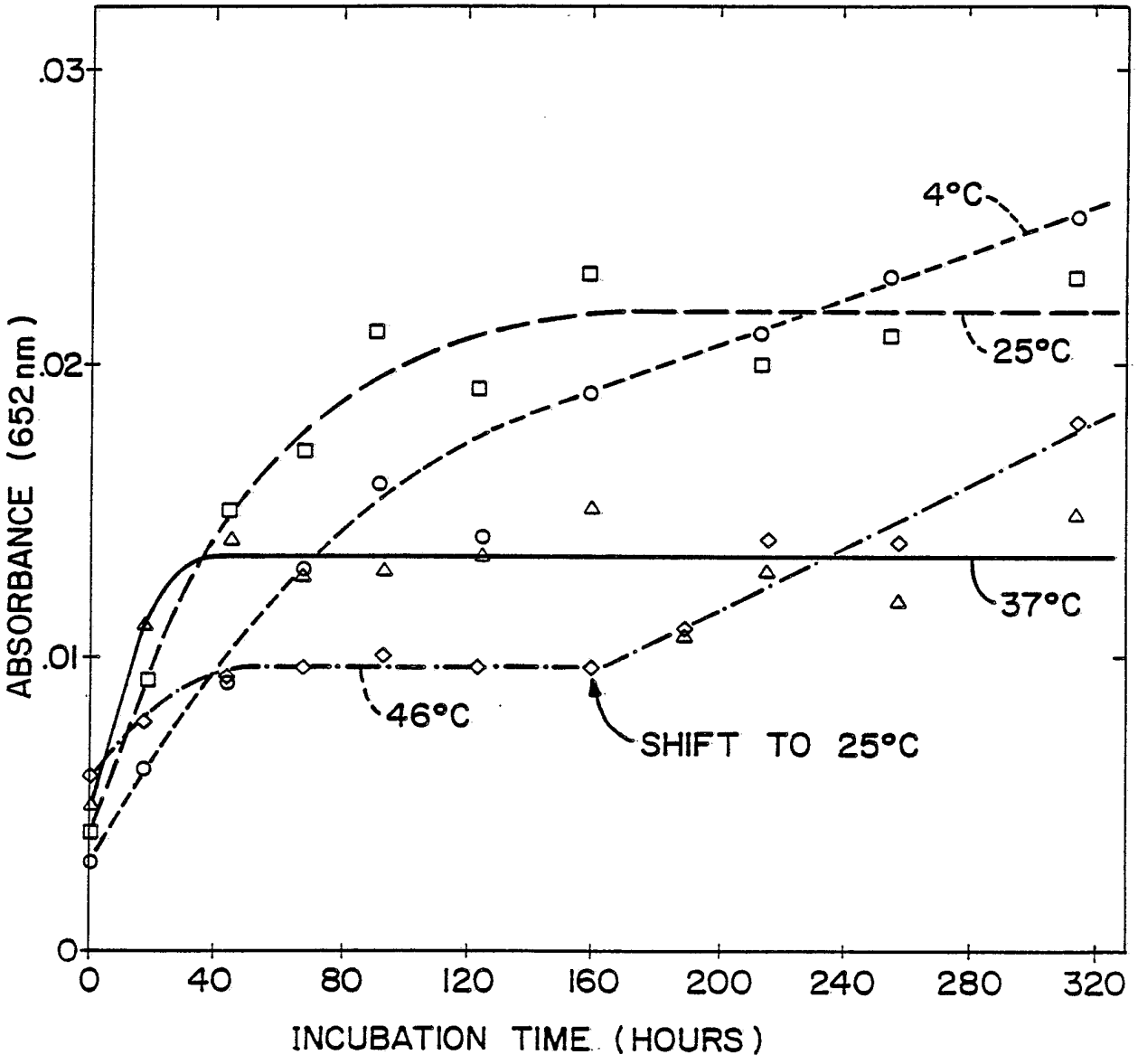
.03
.02
.01
0
ABSORBANCE (652 nm)
0
40
80
120
160
200
240
280
320
INCUBATION TIME (HOURS)
4°C
25°C
37°C
46°C
SHIFT TO 25°C

FIG._5.

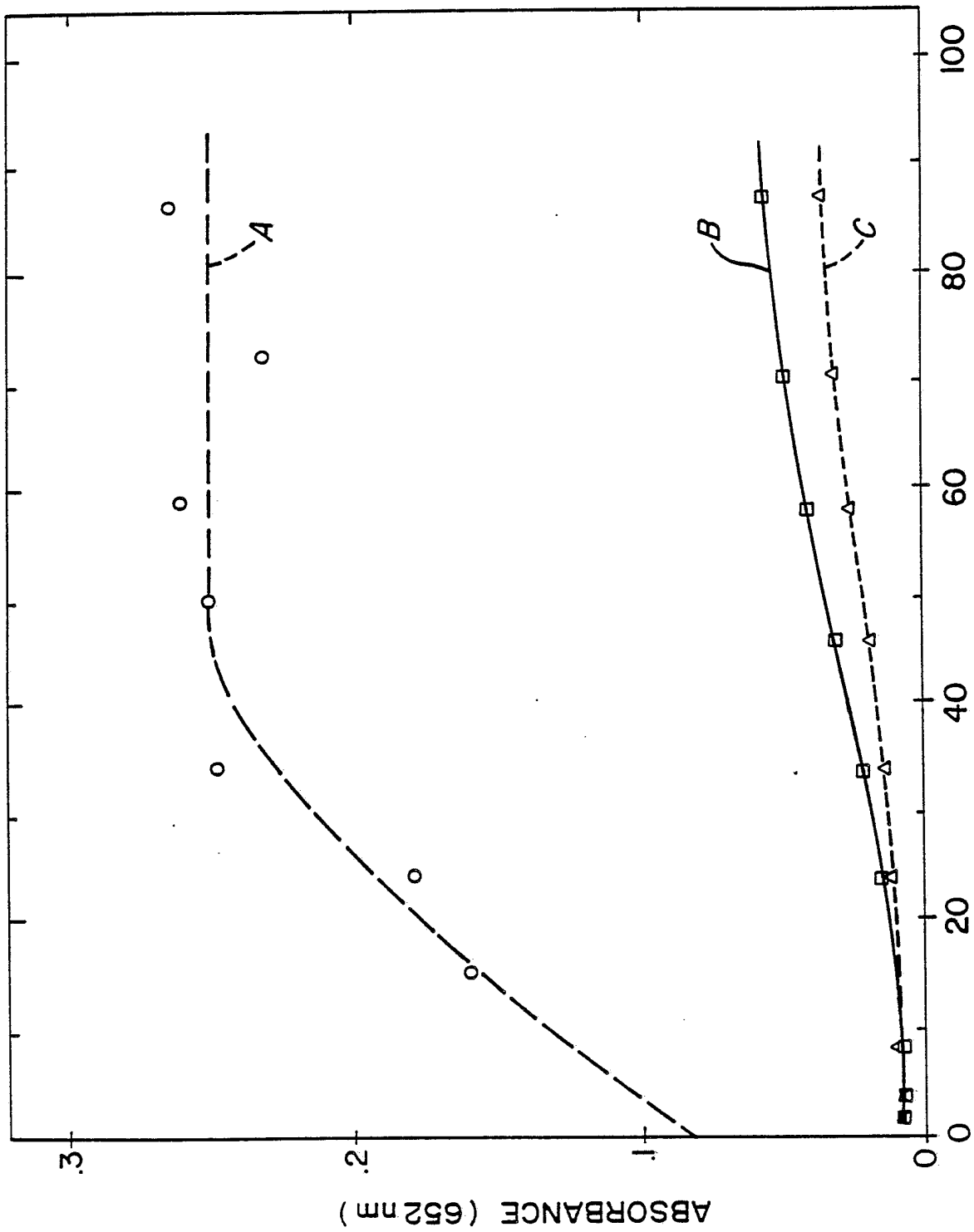
ABSORBANCE (652nm)
0
1
2
3
INCUBATION TIME AT 25°C (HOURS)
0
20
40
60
80
100
A
B
C

FIG._6.

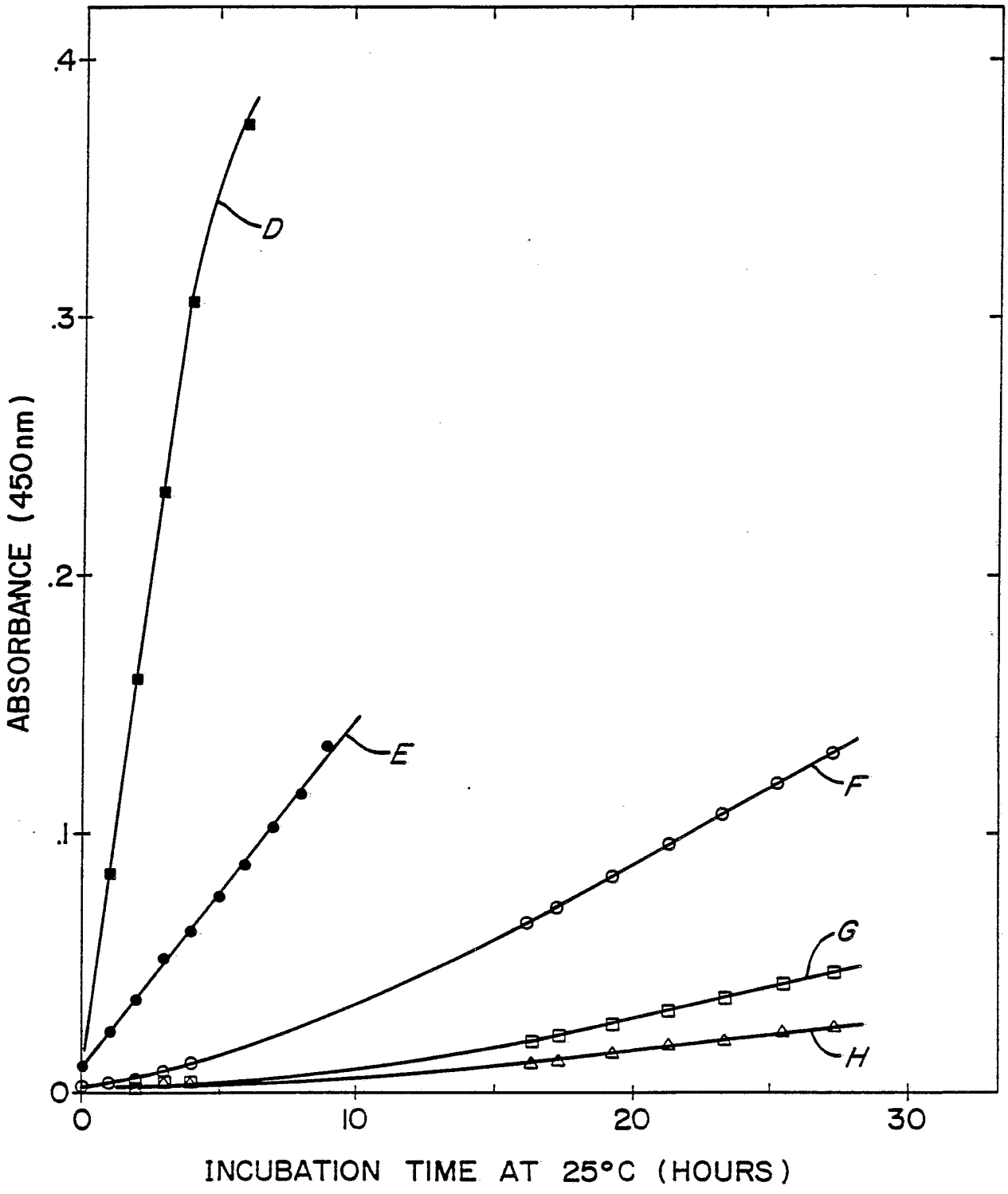
.4
.3
.2
.1
0
ABSORBANCE (450nm)
D
E
F
G
H
0
10
20
30
INCUBATION TIME AT 25°C (HOURS)

FIG._7.

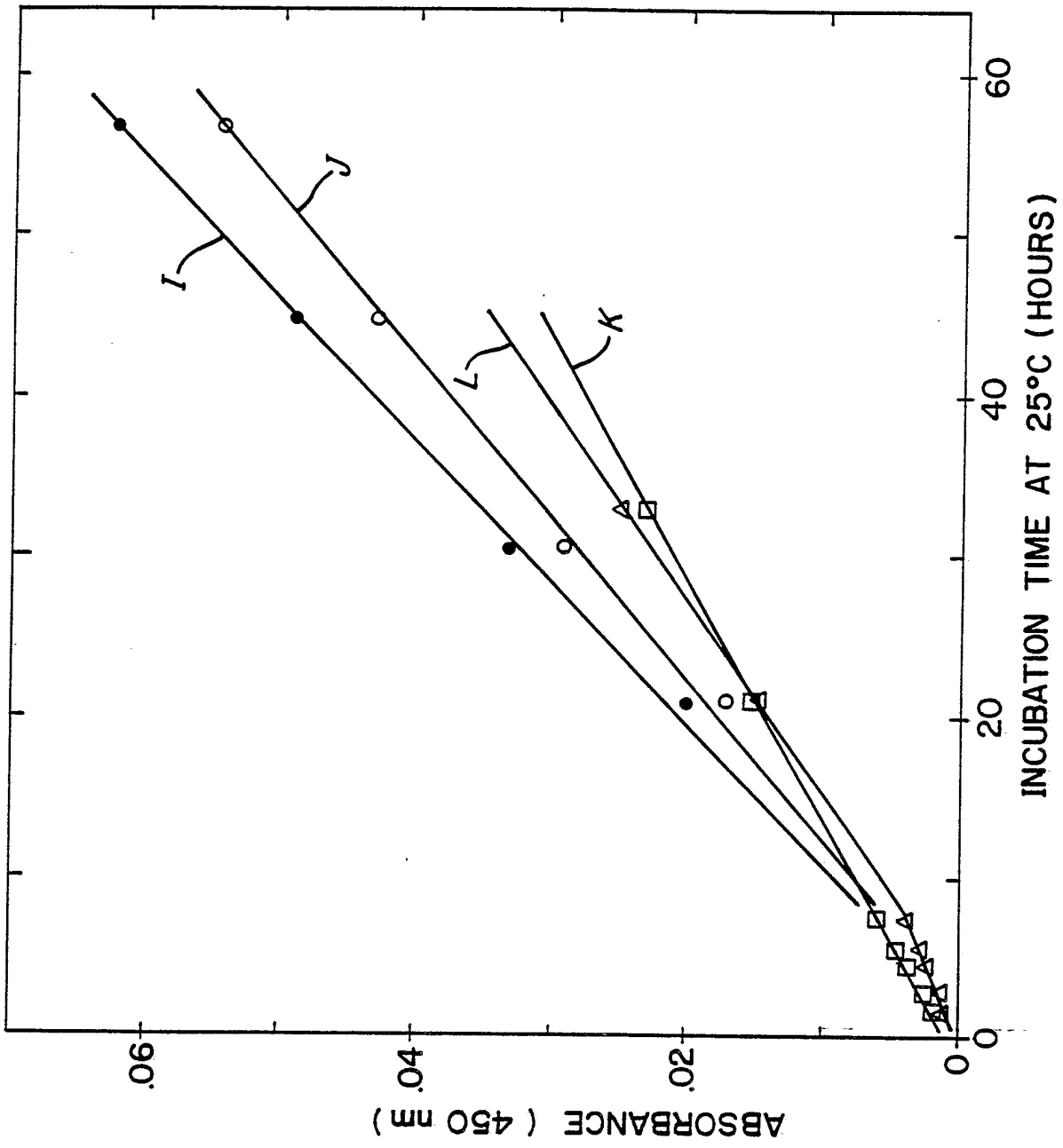
ABSORBANCE (450 nm)
.06
.04
.02
0
0
20
40
60
INCUBATION TIME AT 25°C (HOURS)
I
J
L
K

FIG._8A.

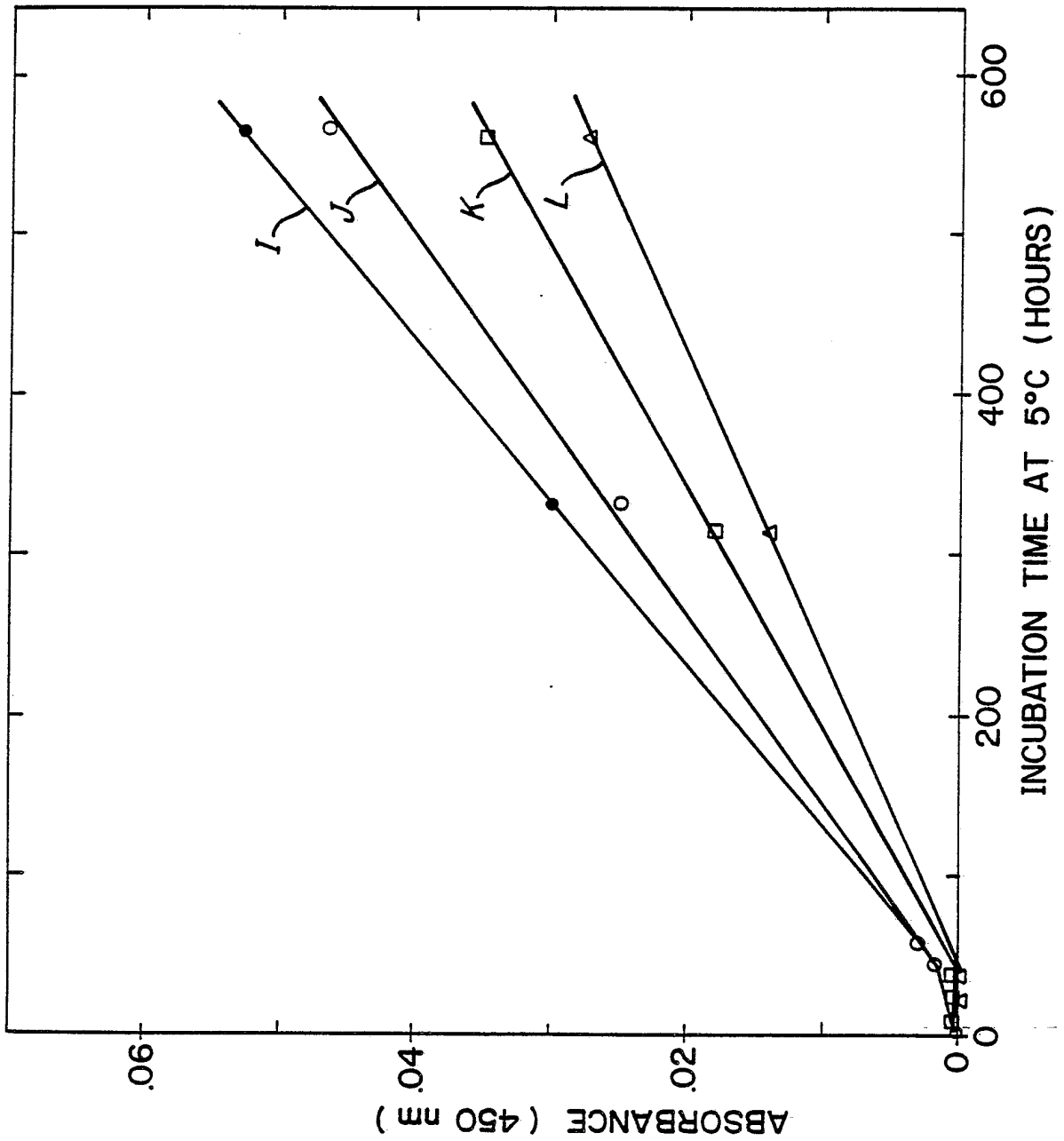
ABSORBANCE (450 nm)
.06
.04
.02
0
0
200
400
600
INCUBATION TIME AT 5°C (HOURS)
I
J
K
L

FIG._8B.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02806

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): G01N 33/72, C12Q 1/28
U.S. Cl. 436/66, 435/28

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/7,28,184,188,810 436/18,66,176,826

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Computer Search: Chemical Abstracts, APS search

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	WO, A, 86/04610 (Thomas) 14 August 1986, see page 2, lines 24-33, page 3, lines 9-28 and page 4, lines 10-16 and 22-27.	1-60
Y	US, A, 4,755,472 (Ismail) 05 July 1988 See column 3, lines 32-54, column 4 lines 53-65 and column 5, lines 1-12.	1-60
Y	WO, A, 87/07718 (Lawrence) 17 December 1987, see page 6, lines 8-15, page 7, lines 9-15, page 11, lines 23-34 and page 13, lines 4-33.	1-60

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21 November 1988	25 JAN 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Esther M. Kepplinger

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A, 253,548 (Ben-Michael) 20 January 1988, see page 3, lines 15-20, page 4, lines 1-20 and page 8, lines 16-19.	1-60
Y	US, A, 4,596,770 (Parham) 24 January 1986, see column 1, lines 41-51 and lines 66-68.	1-60
Y	US, A, 4,333,734 (Fleisher) 08 June 1982, see column 2, lines 50-61 and column 3, lines 27-38.	1-60
Y	US, A, 4,587,220 (Mayambala-Mwanika) 06 May 1986, see column 8, lines 35-43.	1-60